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TRANSMITTAL OF APPEAL BRIEF (Large Entity)	Docket No. 1324.030A

In Re Application Of: **PRINCE *et al.***

Application No.	Filing Date	Examiner	Customer No.	Group Art Unit	Confirmation No.
10/635,725	August 6, 2003	Ford, Vanessa	23405	1645	8868

Invention: **FOULBROOD TREATMENTS**

COMMISSIONER FOR PATENTS:

Transmitted herewith is the Appeal Brief in this application, with respect to the Notice of Appeal filed on: **4/19/2006**

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Kathy Smith Dias
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Dated: **June 23, 2006**

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Applicant:	Prince et al.	Attorney Docket No.	1237
Serial No.:	10/635,725	Group Art Unit:	1645
Filed:	August 6, 2003	Examiner:	Ford, Vanessa
Title:	FOULBROOD TREATMENTS		

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Mail Stop Appeal Brief-Patents, Commissioner For Patents, Board of Patent Appeals and Interferences, P.O. Box 1450, Alexandria, VA 22313-1450 on June 23, 2006.

Kathy Smith Dias
Attorney for Appellants
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Date of Signature: June 23, 2006

To: Mail Stop Appeal Brief-Patents
Commissioner for Patents
Board of Patent Appeals and Interferences
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

APPELLANTS' APPEAL BRIEF TO THE BOARD OF PATENT APPEALS AND INTERFERENCES

This is an appeal from a Final Rejection, mailed October 19, 2005, rejecting claims 1-16, that is, all the claims under consideration in the above-identified application. A Notice of Appeal with a Request for a Three-Month Extension of Time was timely filed on April 19, 2006 and received by the United States Patent and Trademark Office on April 24, 2006. This Appeal Brief

is therefore due on June 24, 2006, and is timely filed. This Brief is accompanied by a check in the amount of $500.00 for payment of the requisite fee set forth in 37 C.F.R. §1.17(c).

The format and content of Appellants' brief is believed to be in compliance with the requirements set forth in 37 C.F.R. §41.37(c). However, if Appellants' brief does not comply with the requirements set forth in 37 C.F.R. §41.37(c), Appellants' request notification of the reasons for noncompliance and the opportunity to file an amended brief pursuant to 37 C.F.R. §41.37(d).

(i) Real Party in Interest

This patent application is assigned to University College Cardiff Consultants Ltd., a company existing under the laws of Great Britain, having offices at 56 Park Place, Cardiff CR1 3XR United Kingdom, by virtue of an Assignment executed on June 14, 2006 and June 15, 2006 and recorded with the United States Patent and Trademark Office at Reel 017827/Frame 0596 on June 22, 2006. Therefore, the real party in interest is University College Cardiff Consultants Ltd.

(ii) Related Appeals and Interferences

To the knowledge of Appellants and Appellants' undersigned legal representative, there are no other appeals or interferences which will directly affect or be directly affected by or have a bearing on the Board's decision in the instant appeal.

(iii) Status of Claims

This patent application was filed as a continuation of a continuation of a PCT International application on August 6, 2003. As filed, the application contained claims 1-16 of which one (1) claim was an independent claim (claim 1).

In an initial Office Action dated April 25, 2005, claims 1-16 were rejected under 35 U.S.C. § 112 second paragraph for failing to particularly point out and distinctly claim the

subject matter which Appellants regard as the invention. Claims 1 and 7-16 were rejected under 35 U.S.C. § 102(b) as being anticipated by Oldroyd et al. (Aust. J. Agric. Res., 1989, 40(3), p. 691-698), Hansen et al. (Tidsskrift for Planteavl, 1988, Vol. 92, No. 1, p. 11-15) and Wilson et al. (Can. J. Microbiol., June 1970, 16(6) 521-526). Claims 1-16 were rejected under 35 U.S.C. § 102(b) as being anticipated by or, in the alternative, under 35 U.S.C. § 103(a) as being obvious over Hoopingarner et al. (American Bee Journal, 1988, Vol. 128, No. 2, p. 120-121). In Appellants' response dated July 25, 2005, claim 1 was amended.

In a second and final Office Action dated October 19, 2005, rejections under 35 U.S.C. 112, second paragraph, 102 (b) and 102/103 from the previous Office Action were withdrawn. Only the rejection of claims 1 and 7-16 under 35 U.S.C. § 102(b) in light of Oldroyd was maintained. In Appellants' Response of January 19, 2006, Appellants' requested withdrawal of the rejection under 35 U.S.C. § 102(b) for the reasons set forth in the response without further amendment.

Appellants received an Advisory Action dated April 11, 2006, which indicated that the rejection of claims 1 and 7-16 under 35 U.S.C. 102(b) was maintained. The Advisory Action also indicated that the rejection of claims 1-16 under 35 U.S.C. §§ 102/103 was withdrawn. However, this rejection was withdrawn previously in response to the initial Office Action.

A Notice of Appeal to the Board of Patent Appeals and Interferences was mailed on April 19, 2006 with a request for a three-month extension of time and fee therefor. The Notice of Appeal was received at the United States Patent and Trademark Office on April 24, 2006.

Although the advisory action of April 11, 2006 indicates for the first time that claims 2-6 are objected to as being dependent on a rejected base claims, there is no suggestion that claims 2-

6 would be allowable if rewritten in independent form to include all the limitations of the rejected base claim.

The status of the claims, according to the Advisory Action appears to be as follows:

Allowed Claims	-	None
Claims Objected to	-	2-6
Claims Rejected	-	1 and 7-16
Claims Appealed	-	1-16.

Appellants are appealing the rejection of claims 1-16.

(iv) Status of Amendments

The claims currently under appeal were presented in an amendment included in Appellants' Response to the initial Office Action dated April 25, 2005 and entered by the Examiner. No amendments have been presented subsequent to this initial amendment. The claims as set out in the Claims Appendix are the claims as amended in response to the initial Office Action.

(v) Summary of Claimed Subject Matter

The invention relates to a method of treating foulbrood disease in bees. The claimed subject matter is a composition for treating foulbrood, the composition comprising an inoculum containing one or more microorganisms that are non-pathogenic to bees for producing a microflora having therapeutic or prophylactic efficacy against the bee disease and an apicultural

delivery vehicle for delivering the inoculum. The apicultural delivery vehicle for delivering the inoculum is selected from a patty, a syrup, a drench, a dusting and a paste.

(vi) Grounds of Rejection To Be Reviewed On Appeal

1. Claims 1-16 are rejected under 35 U.S.C. §102(b) as being anticipated by Oldroyd et al. *(Aust. J. Agric. Res., 1989, 40(3), p. 691-698.)*

(vii) Arguments

1. Rejection of Claims 1-16 under 35 U.S.C. 102(b)

A. APPELLANT'S POSITION

Claims 1-16 stand rejected under 35 U.S.C. 102(b) as being anticipated by Oldroyd et al. *(Aust. J. Agric. Res., 1989, 40(3), p. 691-698.)* Appellants respectfully submit that this rejection is improper, since one or more of Appellants' claimed elements is not disclosed in the cited reference.

B. SUPPORT FOR APPELLANTS' POSITION

"A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." Verdegaal Bros., Inc. v. Union Oil Co. of California, 814 F.2d 628, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987). Anticipation under 35 U.S.C.§102, therefore, requires the presence in a single prior art disclosure of each and every element of a claimed invention.

Appellants' claimed invention, as recited in independent claim 1 of the instant application, recites a composition for the treatment or prophylaxis of a bee disease, the composition comprising: a) an inoculum containing one or more microorganisms that are non-pathogenic to bees for producing a microflora having therapeutic or prophylactic efficacy against the bee disease; and b) an apicultural delivery vehicle for delivering the inoculum to a component of a bee hive or to a bee colony that is susceptible to or infected with the bee disease, whereby a remedial and/or protective microflora is established within the hive or the bee colony. Thus, a primary feature of the claimed composition is an inoculum of one or more microorganisms that are ***non-pathogenic*** to bees.

In order for Oldroyd to anticipate Appellants' claims, therefore, Oldroyd must teach an inoculum containing one or more microorganisms that are non-pathogenic to bees. It does not. The propriety of the rejection of the present claims rests on whether one of skill in the art would, based on the teachings of the Oldroyd reference, in particular, and the literature as a whole, would conclude that *Paenibacillus larvae subsp. larvae* subsp. *larvae* (formerly *B. larvae*) is a non-pathogenic microorganism. Appellants' position is that they would not.

It should be noted that, as evidenced by Heyndrickx et al. (p.990), there have been changes in nomenclature with respect to *Bacillus* taxonomy since the present application was filed. The organism referred to in Oldroyd as *Bacillus larvae* is now known as *Paenibacillus larvae* subsp. *larvae*. Accordingly, the two terms are used herein to refer to the same organism, i.e., the causative agent for American foulbrood.

As a preliminary matter, contrary to the statement on page 3 of the Advisory Action that Appellants do not agree with the definition of non-pathogenic as proposed by the examiner, the definition of the term "non-pathogenic" as used in independent claim 1 is not in dispute. A **non-**

pathogen is a microorganism that is not able to cause disease in a plant, animal or insect. Conversely, a **pathogen** is a microorganism that is able to cause disease.

Relying on the transitional or open claim language "comprising" in independent claim 1, the examiner urges that other components may be present in Appellants' claimed composition. According to the Advisory Action, therefore, the rejection was on the grounds that Oldroyd et al. teach that honeybee colonies were treated with various preparations of oxytetracycline hydrochloride (OTC), an antibiotic to which *B. larvae* is sensitive, at the time of inoculation with *Bacillus larvae* spores. Furthermore, the examiner relies on the statement in Oldroyd et al. that "*B. larvae* was cultured from adult bee samples from colonies...that were American Foubrood (AFB) disease-free at the time of sampling and did not subsequently develop disease signs." From this, the examiner concludes that Oldroyd teaches a composition containing a microorganism that is non-pathogenic to bees, thereby anticipating the composition as claimed by Appellants. There is, however, no evidence in the literature, not even in Oldroyd, to support this conclusion.

Applicants respectfully submit that a proper reading of Oldroyd makes it clear that *B. larvae* is ***not***, as the Action maintains, non-pathogenic to bees. Oldroyd, therefore, cannot anticipate the present invention.

It is quite clear from a survey of the literature, including Oldroyd et al., that *Paenibacillus larvae* subsp. *larvae* is the primary larval pathogen of honey bees (see Evidence appendix.) Oldroyd clearly teaches that which is well known in the art, that is, that American foulbrood (AFB) is caused by the bacterium *Bacillus larvae*.

The literature, including Oldroyd, further teaches that oxytetracyline hydrochloride (OTC) therapy is commonly used to control European foulbrood (EFB) and is used to control AFB in Tasmania and some areas of the United States. One study demonstrated that *Paenibacillus larvae* subsp. *larvae* isolated from Australian sources continue to be very sensitive to OTC and that no resistance to OTC appears to have developed over the past fifteen or sixteen years.

However, a proper reading of Oldroyd would not lead one of skill in the art to conclude that *B. larvae*, even in the presence of OTC, is a non-pathogenic organism useful for the treatment or prophylaxis of a bee disease. Rather, Oldroyd teaches that *Bacillus larvae* is a highly pathogenic organism, even in the presence of OTC, and that frequently treatment with OTC suppresses disease signs.

Oldroyd presents the results of a study of the effect of OTC treatment on American foulbrood. In these studies, honeybee colonies were inoculated with *Bacillus larvae* spores to induce the disease. Additionally, some of the colonies were treated with OTC preparations at two different times: 1) at the same time that the colony was inoculated with the *B. larvae* spores or 2) after American foulbrood disease signs had developed in the colony. Control colonies were inoculated with *B. larvae* to initiate infection, but no OTC was given.

The relevant portions of Oldroyd appear on pages 692 and 693 and reads as follows:

"*Treatment of Colonies with Oxytetracycline Hydrochloride*

Experiment 1: Effect of OTC as a preventative of AFB disease

On 21 November 1986, 10 colonies were inoculated with B. larvae spores. Five of these colonies were treated with 1 g of OTC (Terramycin 50R Pfizer) dissolved in 500 ml of 50% v/v sucrose syrup (S:S) at the time of inoculation. The other five colonies

were treated with 500 ml S:S only."

"**Results**

Experiment 1: Effects of OTC as a Preventative of AFB Disease

All control colonies [emphasis added] inoculated with *B. larvae* spores developed disease signs within 40 days (Fig.1). OTC treatment at the time of inoculation prevented the development of disease signs for 58 days in one hive, 291 days in another hive and two other colonies became diseased in mid-summer, more than 1 year after their inoculation with *B. larvae* spores. ***AFB disease was prevented by OTC treatment at the time of inoculation in 1 colony only*** (Fig.1)."

The results show that all of the colonies that were inoculated with B. larvae in the absence of OTC became diseased. Additionally, four colonies of five (i.e. 80%) inoculated simultaneously with *B. larvae* and OTC developed foulbrood disease. Therefore, even in the presence of an antibiotic to which it is sensitive, *B. larvae* are able to cause disease. Not surprisingly, Oldroyd (abstract) states that *B. larvae* was subsequently cultured from adult bee samples from colonies that did not develop disease signs. These colonies, however, were treated with OTC. Oldroyd does not suggest that these colonies are disease free subsequent to inoculation with *B. larvae* because *B. larvae* is not a pathogen; rather, Oldroyd concludes that these results show that recommended treatments for European foulbrood (EFB), i.e., treatment with OTC, essentially suppress signs of AFB disease.

It is therefore, inconceivable that a person of skill in the art would conclude, based on all the evidence including the teachings of Oldroyd, that *Paenibacillus larvae subsp. larvae* (formerly known as *B. larvae*) is non-pathogenic to bees.

C. CONCLUSION

Appellants respectfully request reversal of the 35 U.S.C. §102(b) rejections of claims 1-16 set forth in the Final Office Action. For the reasons discussed herein, Appellants respectfully submit that the §102(b) rejection based on Oldroyd is clearly erroneous since the cited reference does not teach an inoculum containing one or more microorganisms that are non-pathogenic to bees. Accordingly, reversal of all rejections is respectfully requested.

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(viii) CLAIMS APPENDIX

1. A composition for the treatment or prophylaxis of a bee disease, the composition comprising:

(a) an inoculum containing one or more microorganisms that are non-pathogenic to bees for producing a microflora having therapeutic or prophylactic efficacy against the bee disease; and

(b) an apicultural delivery vehicle for delivering the inoculum to a component of a bee hive, or to a bee colony that is susceptible to or infected with the bee disease, whereby a remedial and/or protective microflora is established within the hive or the bee colony.

2. The composition of claim 1, wherein the apicultural delivery vehicle is selected from:

(a) a patty;

(b) a syrup;

(c) a drench;

(d) a dusting; and

(e) a paste.

3. The composition of claim 2, wherein the apicultural delivery vehicle is a patty selected from:

(a) a sugar patty; and

(b) a sugar and fat extender patty.

4. The composition of claim 2, wherein the apicultural delivery vehicle is a syrup comprising sugar and water.

5. The composition of claim 2, wherein the apicultural delivery vehicle is a dusting comprising sugar.

6. The composition of claim 2, wherein the apicultural delivery vehicle is a paste comprising a pollen substitute.

7. The composition of claim 1, wherein the inoculum comprises one or more microorganism(s) that produce one or more antibiotic(s) active against one or more bee pathogen(s).

8. The composition of claim 7, wherein the antibiotic(s) are active against at least one of *Melissococcus pluton* and *Paenibacillus larvae subsp. larvae* subsp. *larvae*.

9. The composition of claim 7, wherein the antibiotic(s) are bacteriolytic.

10. The composition of claim 7, wherein the antibiotic(s) are the anti-*Melissococcus pluton* and/or the anti-*Paenibacillus larvae subsp. larvae* subsp. *larvae* antibiotic(s) found in *Paenibacillus larvae subsp. larvae* subsp. *pulvifaciens*.

11. The composition of claim 7, wherein the antibiotic(s) are active against one or more of *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas putida*, *Pseudomonas aerugenosa*, *Micrococcus luteus*, *Bacillus cereus*, *Bacillus subtilis*, *Paenibacillus alvei*, *Paenibacillus larvae subsp. larvae* subsp. *larvae*, *Paenibacillus apiarius*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Yersinia enterocolitica*, and *Melissococcus pluton*.

12. The composition of claim 7, wherein the microorganism(s) comprise bacteria.

13. The composition of claim 12, wherein the bacteria comprise bacterial endospores.

14. The composition of claim 13, wherein the bacterial endospores are washed and/or concentrated.

15. The composition of claim 12, wherein the bacteria comprise a *Paenibacillus* spp.

16. The composition of claim 15, wherein the *Paenibacillus* spp. is:

(a) a subspecies of *Paenibacillus larvae*; or

(b) *P. alvei*.

(ix) EVIDENCE APPENDIX

1. Hornitzky, Michael, "Oxytetracycline sensitivity of *Paenibacillus larvae subsp. larvae* subsp. *larvae* isolates," Australian Government Rural Industries Research and Development Corporation, RIRDC Publication No. 05/021, RIRDC Project No. DAN-219A, pp. 1-6 (January 2005).

2. Genersch *et al.*, "Strain- and Genotype-Specific Differences in Virulence of *Paenibacillus larve* subsp. *larvae*, a Bacterial Pathogen Causing American Foulbrood Disease in Honeybees," Applied and Environmental Microbiology, 71(11), pp. 7551-7555 (2005).

3. Alippi *et al.*, "Differentiation of *Paenibacillus larvae subsp. larvae* subsp. *larvae*, the Cause of American Foulbrood of Honeybees, by Using PCR and Restriction Fragment Analysis of Genes Encoding 16S rRNA," Applied and Environmental Microbiology, 68(7), pp. 3655-3660 (2002).

4. Evans *et al.*, "Antagonistic interactions between honey bee bacterial symbionts and implications for disease," BMC Ecology, 6(4), pp. 1-9, (2006).

5. Neuendorf *et al.*, "Biochemical characterization of different genotypes of *Paenibacillus larvae subsp. larvae* subsp. *larvae*, a honey bee bacterial pathogen," Microbiology, 150, pp. 2381-2390 (2004).

6. "*Paenibacillus larvae subsp. larvae* subsp. *larvae*," from DSMZ-List of Microbial Species: *Paenibacillus larvae subsp. larvae* subsp. *larvae* (Bacteria) by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

7. "American Foulbrood," VITA, pp. 1-3, http://www.beekeeping.com/vita/disease/American.htm.

8. Honey Bee Research, pp. 1-2, http://www.ars.usda.gov/research/projects/projects.htm

(x) RELATED PROCEEDINGS APPENDIX

NONE

Australian Government
Rural Industries Research and Development Corporation

Oxytetracycline sensitivity of *Paenibacillus larvae.* subsp. *larvae* isolates

A report for the Rural Industries Research and Development Corporation

by Michael Hornitzky

January 2005

RIRDC Publication No 05/021
RIRDC Project No DAN-219A

ISBN 1 74151 120 8
ISSN 1440-6845

Oxytetracycline sensitivity of Paenibacillus larvae. *subsp.* larvae *isolates*
Publication No. 05/021
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Published in January 2005
Printed on environmentally friendly paper by Canprint

Foreword

American foulbrood (AFB), caused by the bacterium *Paenibacillus larvae* subsp. *larvae*, is a major bacterial honey bee disease which causes significant economic loss to the beekeeping industry in Australia and around the world. Oxytetracycline hydrochloride (OTC) has been used to treat AFB for 4 decades. However, in recent years OTC-resistant strains have emerged in the United States of America, Canada and Argentina. Although in Australia OTC is only used in Tasmania to treat AFB it is important for the Australian beekeeping industry to know whether OTC-resistant *P. l. larvae* strains are in Australian bees and whether imported honey contains OTC-resistant *P. l. larvae*. The presence of OTC-resistant *P. l. larvae* in Australian bees will influence the choice of future control options for the control of AFB. It is also possible that OTC-resistant *P. l. larvae* may transfer this resistance to *M. pluton*, the cause of European foulbrood, another important bacterial disease of honey bees in Australia.

The aim of this study was to; (i) acquire a range of *P. l. larvae* from around Australia and determine the minimum inhibitory concentration (MIC) of OTC to these isolates, (ii) determine the MIC of OTC to *P. l. larvae* isolates obtained from imported honey and (iii) compare current OTC sensitivities to the MICs of *P. l. larvae* isolates collected in the late 1980s.

This project was funded from industry revenue which is matched by funds provided by the Australian Government.

This report, an addition to RIRDC's diverse range of over 1200 research publications, forms part of our Honeybee R&D program, which aims to improve the productivity and profitability of the Australian beekeeping industry.

Most of our publications are available for viewing, downloading or purchasing online through our website:

- downloads at www.rirdc.gov.au/fullreports/index.html
- purchases at www.rirdc.gov.au/eshop

Tony Byrne
Acting Managing Director
Rural Industries Research and Development Corporation

Acknowledgments

We thank Aida Ghalayini, Technical Officer for her excellent laboratory work. We thank Wendy Ward, Department of Primary Industries, Animal Research Institute, Yeerongpilly, Queensland and Nicky Buller, Agriculture Western Australia, Animal Health laboratories, South Perth, Western Australia for submitting American foulbrood positive smears. We also thank the beekeepers and Capilano Honey Ltd who kindly sent samples of honey and larval smears for analysis.

Contents

Executive Summary

American foulbrood (AFB), caused by *Paenibacillus larvae* subsp. *larvae*, is considered to be the most important bacterial disease of honey bees in Australia. In many countries oxytetracycline hydrochloride (OTC) is used to treat the disease. On mainland Australia AFB is controlled by the incineration of infected hives or the irradiation of hive material from diseased hives. Tasmania is the only state which permits treatment with OTC.

In recent years OTC-resistant *P. l. larvae* have emerged in the United States of America, Canada and Argentina. There is no information on the OTC sensitivity of *P. l. larvae* in Australian bees and whether honey imported from overseas (Argentina) contains OTC-resistant *P. l.* larvae. This information is important as it has a bearing on future control options for bacterial honey bee diseases in Australia.

This study has demonstrated that *P. l. larvae* isolated from Australian sources are very sensitive to OTC and that no resistance to OTC appears to have developed over the past 15/16 years. Most isolates from imported honey had higher minimum inhibitory concentrations for OTC than Australian isolates but the difference was so minor that they would all still be considered to be very sensitive to OTC. This indicates that honey imported from Argentina has not been a significant source of OTC-resistant *P. l. larvae*.

1. Introduction

American foulbrood (AFB), caused by *Paenibacillus larvae* subsp. *larvae*, is a major bacterial honey bee disease which causes significant economic loss to the beekeeping industry in Australia and around the world. Oxytetracycline hydrochloride (OTC) has been used to treat AFB for 4 decades. However, in recent years OTC resistant strains have emerged in the United States of America, Canada and Argentina (Miyagi et al, 2000). Although in Australia OTC is only used in Tasmania to control AFB it is important for the Australian beekeeping industry to know whether OTC-resistant *P. l. larvae* strains are in Australian bees as the presence of such organisms in the Australian bee hives will influence the choice of future control options for the control of AFB.

It is also possible that OTC-resistant *P. l. larvae* may transfer this resistance to *Melissococcus pluton* (the cause of European foulbrood). OTC is the only antibiotic registered in Australia for use against EFB. The development of OTC- resistant *M. pluton* would have a severe impact on the profitability of bee farming in Australia and necessitate the introduction of an alternative antibiotic to treat EFB.

2. Objective

The aim of this study was; (i) to acquire a range of *P. l. larvae* from around Australia, (ii) to determine the minimum inhibitory concentration (MIC) of OTC to these isolates, (iii) to determine the MIC of OTC to isolates obtained from Argentinean honey and (iv) to compare current OTC sensitivities to the MICs of *P. l. larvae* isolates collected in the late 1980s.

3. Methodology

3.1 *Paenibacillus larvae* subsp. *larvae* isolates

P. l. larvae isolates were obtained by culture of honey samples and larval smears submitted by beekeepers in Australia or by veterinary laboratories in Queensland, Western Australia and Tasmania (Hornitzky and Anderson, 2002). Isolates were also cultured from imported honey collected from supermarket shelves and honey samples from individual Argentinean beekeepers provided by a honey packing plant. Isolates from the *P. l. larvae* culture collection held at the Elizabeth Macarthur Agricultural Institute, Menangle, New South Wales were also used for comparative purposes to determine whether any OTC resistance had developed during the past 15 or 16 years.

3.2 Determining the minimum inhibitory concentration of OTC for *P. l. larvae* isolates

The MICs of OTC to *P. l. larvae* isolates were determined using the agar dilution method. *P. l. larvae* cultures which were initially cultured on 7% sheep blood agar (SBA) (Hornitzky and Clark, 1991) and incubated at 37°C for 2 days in an atmosphere containing 5% CO_2 were used as inocula.

Inocula were prepared by touching a single colony from a pure (second subculture) culture of *P. l. larvae* with a bacteriologists loop. A single line was then streaked on brain heart infusion agar plates (5 lines per plate). Plates were incubated as described previously.

Isolates were considered to be sensitive to a specific OTC concentration if their growth was markedly or completely inhibited by that concentration (Stokes and Ridgway, 1980).

4. Results

4.1 *Paenibacillus larvae* subsp. *larvae* isolates

Seventy nine *P. l. larvae* isolates were used in this study. These consisted of isolates from New South Wales, Queensland, Western Australia, Victoria, South Australia, Tasmania, imported honey purchased from supermarkets, imported honey from individual Argentinean beekeepers and Australian isolates collected in 1988/1989 (Table 1). These isolates were identified as *P. l. larvae* as described by Hornitzky and Anderson (2002).

4.2 The MIC of OTC for *P. l. larvae* isolates

Preliminary MIC assays indicated that the test isolates belonged to two groups; those that were sensitive to 0.1 µg/ml of OTC and those that were resistant to 0.1µg/ml. Following further preliminary studies the first group of isolates were then tested on two separate occasions with 0.01, 0.02, 0.03, 0.04 and 0.05 µg/ml of OTC. Those isolates resistant to 0.1µg/ml were tested on two separate occasions to 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 µg/ml. Of the 79 isolates used in this study one isolate had a MIC of OTC of 0.03 µg/ml, 56 had a MIC of 0.04 µg/ml and seven had an MIC of 0.05 µg/ml. Four had a MIC of 0.5 µg/ml and 11 had a MIC of 0.6 µg/ml. All these 15 isolates except one were cultured from imported honey or honey which contained blends which also included imported honey. The one exception was an isolate cultured from a honey sample from a Victorian beekeeper (Table 1).

Table 1. Sensitivity of *P. l. larvae* isolates to OTC

		No of isolates sensitive to OTC (µg/mL)				
Origin	No of isolates	0.03	0.04	0.05	0.5	0.6
New South Wales	15	0	14	1	0	0
Queensland	16	1	15	0	0	0
Western Australia	7	0	7	0	0	0
Victoria	7	0	5	1	0	1
South Australia	3	0	3	0	0	0
Tasmania	4	0	4	0	0	0
Imported honey (ex supermarket)	13	0	6	0	1	6
Imported honey (ex specific beekeepers)	7	0	0	0	3	4
Old cultures (1988/89)	7	0	2	5	0	0
Total	**79**	**1**	**56**	**7**	**4**	**11**

5. Discussion

OTC has been used, for up to 40 years, for the treatment of AFB in a number of countries including the United States of America, Canada and Argentina. However, in recent years there has been an emergence of OTC-resistant *P. l. larvae* strains in these three countries. The degree of sensitivity/resistance to OTC reported by Miyagi et al (2000) has been quite variable ranging from >32.00 µg/ml for the American UCD P-MN spore isolate, to 10-15 µg/mL for Argentinian isolates to <1.00 µg/ml for the susceptible American strain (NRRL B-3650). However, there is no information on the sensitivity of Australian *P. l. larvae* to this antibiotic.

In this study all the isolates, except one, cultured from Australian brood or honey samples were sensitive to very low concentrations of OTC (from 0.03 to 0.05 µg/ml; Table 1). These isolates were of the same order of sensitivity as those in the late 1980s indicating that *P. l. larvae* has not developed any resistance over the past 15 years. All isolates cultured from the imported honey samples from beekeepers from Argentina were susceptible to OTC but at about 10 times the concentrations required to inhibit the growth of the Australian isolates. Despite the increased concentration of OTC required to inhibit the isolates from the Argentinean honey all these isolates would still be considered to be very sensitive to OTC. It has previously been reported that Australian isolates of *Melissococcus pluton* (the cause of European foulbrood) are sensitive to 1 or 2 µg/ml (Hornitzky and Smith, 1999). This is about 4 times the concentration required to inhibit Argentinean *P. l. larvae* isolates detected in this study.

There was one isolate cultured from a Victorian honey sample which had an MIC equivalent to those cultured from all the honey samples submitted from the Argentinean beekeepers. Although this isolate is considered to be sensitive to the OTC it indicates that there may be transfer of *P. l. larvae* from imported honey to Australian honey bee colonies.

During this study it was noticed that as the more the *P. l. larvae* isolates were subcultured the more quickly they became adapted to increased concentrations of OTC. To avoid the artificial detection of OTC-resistant *P. l. larvae* isolates the test cultures were not subcultured more than twice after culture from the stock culture agar slope. The marginally increased resistance of the 1988/89 isolates to OTC may have been a function of their increased subculture rates although there are no records of how many times they had been subcultured before the assays in this study were undertaken.

In summary this snapshot study has demonstrated that *P. l. larvae* isolates of Australian origin are very sensitive to OTC. Isolates cultured from honey imported from Argentina are also sensitive to OTC but at a level approximately ten fold above the sensitivity determined for Australian isolates. The single *P. l. larvae* isolate with a MIC of 0.6 µg/ml cultured from a Victorian beekeeper's honey sample suggests that an Argentinean *P. l. larvae* strain has infected an Australian honey bee colony. It would be useful to continue monitoring of this type to determine if the OTC sensitivity of *P. l. larvae* in Australian honey bee colonies changes.

6. Recommendations

That *P. l. larvae* isolates from Australian bees and imported honey continue to be monitored for their sensitivity to OTC.

That imported honey samples be cultured for *M. pluton* (the cause of EFB) to determine whether resistant *M. pluton* strains have been imported into Australia.

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APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 2005, p. 7551–7555 Vol. 71, No. 11
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.11.7551–7555.2005

Strain- and Genotype-Specific Differences in Virulence of *Paenibacillus larvae* subsp. *larvae*, a Bacterial Pathogen Causing American Foulbrood Disease in Honeybees

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Received 4 May 2005/Accepted 23 June 2005

Virulence variations of *Paenibacillus larvae* subsp. *larvae*, the causative agent of American foulbrood disease of honeybees, were investigated by analysis of 16 field isolates of this pathogen, belonging to three previously characterized genotypes, as well as the type strain (ATCC 9545) of *P. larvae* subsp. *larvae*, with exposure bioassays. We demonstrated that the strain-specific 50% lethal concentrations varied within an order of magnitude and that differences in amount of time for the pathogen to kill 100% of the infected hosts (LT_{100}) correlated with genotype. One genotype killed rather quickly, with a mean LT_{100} of 7.8 ± 1.7 days postinfection, while the other genotypes acted more slowly, with mean LT_{100}s of 11.2 ± 0.8 and 11.6 ± 0.6 days postinfection.

The gram-positive, spore-forming bacterium *Paenibacillus larvae* subsp. *larvae* is the primary bacterial pathogen of honeybee brood and the causative agent of American foulbrood disease (AFB). AFB is a cosmopolitan disease and one of the major threats to beekeeping, since it is highly contagious and able to kill affected colonies. Hence, it causes considerable economic loss to beekeepers worldwide.

Spores are the only infectious form of this organism. Larvae become infected by ingestion of spore-contaminated honey. During the first 12 to 36 h after hatching, larvae are most susceptible to infection, with a dose of about 10 spores or fewer being sufficient to successfully infect and finally kill a larva (19, 20). The clinical symptoms of AFB are typical, with the brown, viscous larval remains forming a ropy thread when drawn out with a matchstick. The decaying larvae desiccate into hard scales, consisting of millions of bacterial spores.

So far, studies addressing differences in the outcomes of AFB have only focused on aspects of host tolerance (2, 3, 9, 15, 16, 20) but have neglected the possibility of variation in virulence among different strains of *P. larvae* subsp. *larvae*. Hence, although *P. larvae* subsp. *larvae* is an important pathogen, its pathogenic mechanism and virulence factors remain elusive. Molecular studies addressing these questions are hampered not only by the lack of genomic tools for this organism but also because no thorough phenotypic studies relating to the virulence of this pathogen exist.

Recently, we identified different genotypes of *P. larvae* subsp. *larvae* (7, 12) via repetitive-element PCR fingerprinting with primers ERIC, MBO REP1, and BOX A1R (18). Biochemical fingerprinting of these genotypes by a carbon source test revealed that they differ in their metabolic patterns (12). In addition, only one of the described genotypes, genotype *AB*, was shown to harbor plasmid DNA (12). Here we present data on the further characterization of these genotypes in terms of virulence. We demonstrate for the first time that different strains of *P. larvae* subsp. *larvae* clearly differ in virulence and that some of these differences are genotype specific. The impact of our findings for the transmission of the pathogen is discussed below.

Isolation and identification of bacterial isolates. The *P. larvae* subsp. *larvae* type strain, ATCC 9545 (obtained from the American Type Culture Collection through U. Rdest, Biozentrum der Universität Würzburg), and 16 German field isolates of *P. larvae* subsp. *larvae* isolated from honey samples originating from clinically diseased, AFB-positive hives used in this study are listed in Table 1. Field isolates were sampled during the course of foulbrood monitoring programs between 2000 and 2004. Cultivation and identification of the isolates were performed as described previously (7, 12). Detailed biochemical and genetic analyses of the reference strain and the field strains 00-087, 01-455, 02-130, 03-125, 01-440, 02-113, 02-120, 03-159, 00-1163, 03-522, and 03-525 have been already reported (10, 12). For genetic fingerprinting of the five additional isolates used in this study, previously described techniques were employed (7, 12). All chemicals and media for microbiological work were obtained from Oxoid, Germany.

Preparation of defined spore suspensions for exposure bioassays. For the preparation of spore suspensions containing a defined concentration of CFU, around 100 *P. larvae* subsp. *larvae* colonies per strain resuspended in 300 μl brain heart infusion broth were used to inoculate the liquid part of Columbia sheep blood agar slants and incubated at 37°C for 10 days. Subsequently, the liquid part was analyzed by phase-contrast microscopy for the absence of vegetative cells. Spore concentrations were determined by cultivating serial dilutions on Columbia sheep blood agar plates as described previously (7, 12) and calculating the mean numbers of colonies grown on five plates. Suspensions were adjusted to a concentration of 1×10^7 CFU ml^{-1}. Spore suspensions were stored at 4°C.

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TABLE 1. List of strains used in this study, with values for LC_{50} and LT_{100}

P. larvae subsp. *larvae* strain	Yr of isolation	Genotype	Estimated LC_{50} (CFU ml^{-1} larval diet)	LT_{100} (days p.i.)[a]	
				Min/max	Mean ± SD
00-1163	2000	*AB*	200	7/10	8.3 ± 1.1
03-194	2003	*AB*	260	7/8	7.3 ± 0.6
03-200	2003	*AB*	550	6/10	8.8 ± 1.9
03-522	2003	*AB*	600	7/10	8.3 ± 1.5
03-525	2003	*AB*	620	7/10	8.0 ± 1.0
04-309	2004	*AB*	≪100	6/7	6.6 ± 1.1
01-440	2001	*Ab*	≪100	10/11	10.7 ± 0.6
02-113	2002	*Ab*	200	11/12	11.8 ± 0.4
02-120	2002	*Ab*	110	10/12	11.0 ± 1.0
03-159	2003	*Ab*	270	11/12	11.7 ± 0.6
03-189	2003	*Ab*	800	10/11	10.5 ± 0.5
00-087	2000	*ab*	≪100	11/12	11.3 ± 0.6
01-455	2001	*ab*	550	12/12	12.0 ± 0.0
02-130	2002	*ab*	500	11/13	12.0 ± 1.0
03-119	2003	*ab*	175	11/12	11.7 ± 0.6
03-125	2003	*ab*	330	11/12	11.3 ± 0.6
ATCC 9545[b]		*aβ*	200	10/12	11.3 ± 0.8

[a] p.i., postinfection; min, minimum; max, maximum.
[b] Reference strain.

Exposure bioassays for the investigation of the virulence of *P. larvae* subsp. *larvae* isolates. The virulence levels of different *P. larvae* subsp. *larvae* isolates were determined by exposure bioassays, which, in contrast to injection bioassays, require all of the steps in pathogenesis (17). For experimental infection, worker larvae collected from different colonies of *Apis mellifera carnica* maintained in the apiary at the Institute for Bee Research in Hohen Neuendorf, Germany, were reared in 24-well tissue culture plates according to the method of Peng and coworkers (14), with a modified larval diet consisting of 3% (wt/vol) fructose, 3% (wt/vol) glucose, and 66% (vol/vol) royal jelly (purchased from a local beekeeper) in sterile double-distilled water. Worker larvae of the first larval instar (around 12 h of age) were used throughout the experiments. Since mean weights differ significantly between different age groups (2), ages of the larvae were estimated by size. For infection, final concentrations of 100, 300, 500, 1,000, and 2,000 CFU ml^{-1} larval diet were adjusted by using a working solution of 1×10^5 CFU ml^{-1}. The infectious larval diet was fed to the larvae for the first 24 h after grafting. Thereafter, normal larval diet was used for feeding. Control larvae were fed with normal larval diet throughout the entire larval stages.

Three groups of 10 larvae of the first larval instar were grafted into three wells filled with larval diet (normal or infectious) by using a special grafting tool (Graze Bienenzuchtgeräte, Germany) to avoid injuring the larvae. These three groups on one plate were treated as one replicate. One experiment consisted of four replicates: three infected groups and one noninfected control. For genotypes *ab*, *Ab*, and *AB*, five, five, and six strains, respectively (Table 1), with three to five concentrations each, were tested. For the reference strain, ATCC 9545, three concentrations were tested and the assays were performed three times.

Each day, the larvae were taken out of the incubator and examined under a stereo microscope. Larvae were classified as dead when they stopped respiration, lost their body elasticity, or developed marked edema and when they displayed color changes to grayish or brownish. The number of dead larvae was recorded, and surviving larvae were transferred to new wells freshly filled with food. After defecation, i.e., after clear uric acid crystals and light-yellow excretions could be observed in the remaining diet, engorged larvae were transferred into pupation plates lined with Kimwipes tissue, where they underwent the stages of pupal development. While noninfected larvae successfully underwent metamorphosis, infected larvae rarely developed beyond the stage of engorged larvae or prepupae. For the purpose of this study, mortality occurring after defecation, i.e., in the pupation plates, was referred to as "mortality after cell capping," since the time of defecation (i.e., opening of the gut, marking the transition from larval to pupal development and the beginning of metamorphosis) of in vitro-reared larvae represents the time of capping in the colony (14). Dead animals were classified as dead from AFB only when vegetative *P. larvae* subsp. *larvae* could be cultivated from the larval remains. On no occasion was *P. larvae* subsp. *larvae* cultivated from remains of dead control animals. Experiments with a mortality exceeding 15% in the control group were excluded, as were experiments where the "natural" mortality (larval death but no growth of *P. larvae* subsp. *larvae*) in the infected groups was higher than 15%. The first three experiments were performed three times to demonstrate that the concentration-mortality relationship was reproducible.

Determination of the LC_{50}s of different strains. A common measure of virulence from exposure bioassays is the 50% lethal concentration (LC_{50}), the respective concentration it takes to kill 50% of the hosts tested (17). To obtain this measure, for each strain and concentration the percentage of AFB-dead larvae was calculated and plotted against the spore concentration used for infection. Results showed a clear positive concentration-mortality relationship (Fig. 1). From the obtained graphs, an LC_{50} for each isolate was estimated. The LC_{50}s varied within an order of magnitude between the isolates and revealed no correlation with genotype (Table 1). The most virulent strains (00-087, 01-440, and 04-309), in terms of spore count, killed 50% of the larvae with less than 100 CFU ml^{-1} larval diet, whereas it took the least virulent strain (03-189) around 800 CFU ml^{-1} larval diet to kill 50% of the larvae (Table 1).

It is well-known from field observations that some colonies show no clinical symptoms despite a high spore concentration contaminating the honey, while others exhibit clinically diseased brood although the spore concentration detectable in the honey is low (8). So far, these differences have been explained by differences in host tolerance and hygienic behavior of honeybees (2, 3, 8, 9, 15, 16, 19, 20). Indeed, a study directly comparing a susceptible bee line with a resistant bee line revealed that differences between bee strains might account for a factor of 2 in the spore dose needed for causing clinical symptoms (9).

Our results indicate another important factor involved in determining the outcome of AFB infections in honeybee colonies: variation in pathogen virulence. The LC_{50}s of different *P. larvae* subsp. *larvae* strains, determined by exposure bioassays, varied with a factor of 10, suggesting that the impact of *P. larvae* subsp. *larvae* virulence on the outcome of an AFB

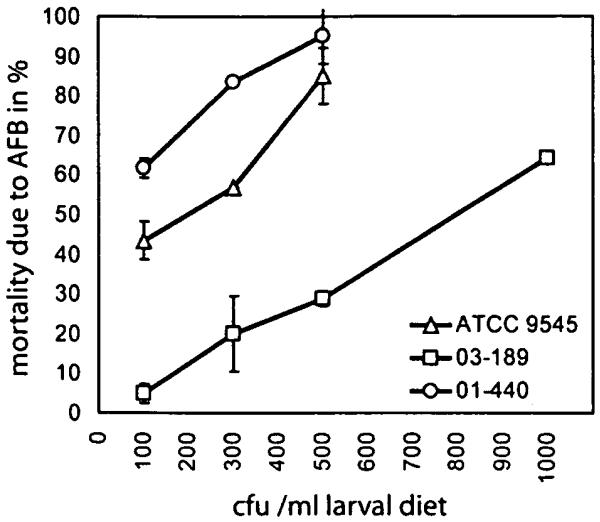

FIG. 1. Determination of LC_{50}s. Representative results obtained for the reference strain, ATCC 9545, and strains 03-189 and 01-440 are shown. Mortality due to AFB was determined as described in the text, and the percentage of AFB-dead larvae was calculated for each concentration (100% = 30 larvae). Experiments were performed three times. The mean values ± standard deviations of mortality are plotted against the spore concentrations to determine the strain-specific LC_{50}s.

infection is much greater than the influence from bee tolerance to infection, reported to vary with a factor of 2 (9).

Determination of the LT_{100}s of different *P. larvae* subsp. *larvae* isolates. Another valid measure of virulence is the time it takes the pathogen to kill 50 or 100% of the infected hosts (LT_{50} or LT_{100}, respectively) (17). To obtain the time course of infection (Fig. 2) and determine the LT_{100} (Table 1), the cumulative proportion of AFB-dead larvae per day was calculated for each replicate and plotted against time. Survivors were excluded from this calculation (17). Results showed that progression of the disease and time of larval death had only a minor, nonsignificant, negative correlation in some cases (Fig. 2B and C). For most of the strains tested and especially for genotype *AB* (Fig. 2A), the time course of infection was not even influenced by the spore concentration. For *P. larvae* subsp. *larvae* genotype *AB*, classical sigmoid curves were obtained, while for the other genotypes, the time course of infection revealed a biphasic curve progression in which two exponential phases of mortality were separated by a phase of reduced mortality between day 5 and day 9 postinfection. For all genotypes, the first dead larvae appeared between day 3 and day 5 postinfection. Larvae infected with genotype *AB* did not survive longer than 10 days postinfection (Table 1) and died rather quickly, with a mean LT_{100} of 7.8 ± 1.7 days postinfection (Table 2). In contrast, the other genotypes killed with mean LT_{100}s of 11.2 ± 0.8 (*Ab*), 11.6 ± 0.6 (*ab*), and 11.3 ± 0.8 ($a\beta$) days postinfection (Table 2), and infected larvae survived at least until day 10 postinfection (Table 1). The genotype-specific differences in disease progression became even more evident by comparing the mean cumulative mortalities of the genotypes (Fig. 2D).

Determination of time of death with respect to cell capping. Capping of the cells is a critical time for both the hygienic behavior of the bees and the time of death due to AFB (1). Therefore, we additionally evaluated the results by choosing this point of time as a threshold. Since the postdefecation period corresponds to the postcapping period, the time of larval defecation was used as the indicator for the beginning of metamorphosis and the time point of cell capping under normal colony conditions (14). For each strain, the mean number of AFB-dead larvae which died after defecation in the pupation plates was calculated over the entire concentration range tested and expressed as a percentage of the total number of AFB-dead larvae. Based on these values, the mean number of larvae that died from AFB after defecation was calculated for each genotype. Results showed that proportion of larvae that died after capping correlated with genotype (Table 2). Only 5.4 ± 3.2% of larvae infected with *P. larvae* subsp. *larvae* genotype *AB* survived until after capping, whereas 26.6 ± 7.3, 20.2 ± 6.3, and 26.3 ± 2.8% of larvae infected with genotypes *Ab*, *ab*, and $a\beta$, respectively, died after capping. Statistical evaluation of these data by one-way analysis of variance ($df = 3$, $F = 14.06$, $P = 0.0002$) followed by a post hoc test (Newman-Keuls test) revealed no significant differences in time postinfection for larval mortality between genotypes *ab* and *Ab* ($P = 0.29$), *ab* and $a\beta$ ($P = 0.14$), or *Ab* and $a\beta$ ($P = 0.98$). In contrast, differences between the three genotypes *Ab*, *ab*, and $a\beta$ and the genotype *AB* for when *P. larvae* subsp. *larvae*-infected larvae died were highly significant, with *P* values of 0.0006, 0.002, and 0.0004, respectively.

Our data demonstrate that *P. larvae* subsp. *larvae* genotype *AB* killed infected larvae much more quickly and earlier than the other genotypes. Therefore, genotype *AB* was the most virulent genotype with respect to disease progression at the level of the individual larva. It can be hypothesized that the highly virulent (with respect to disease progression at individual larva level) *AB* genotype may be less virulent at colony level. The killing of most infected larvae before capping of the cells is likely to allow removal of diseased brood by nursing bees, with fewer bacterial spores produced and spread within the colony than with slower-acting strains that allow the bees to cap the cells before the host is killed. A parallel is found with *Apis cerana*, an Asian species of honeybee also susceptible to *P. larvae* subsp. *larvae* infections. Experiments have demonstrated that most of a *P. larvae* subsp. *larvae*-infected brood is removed before the cells are sealed for pupation, and as a consequence, colonies of *A. cerana* clinically diseased with AFB are less frequent than diseased colonies of *A. mellifera* in the same area (4). However, the hypothesis that the variations in virulence demonstrated by exposure bioassays translate to variations in virulence at colony level remains to be investigated and verified in the field.

Against the background of our results, reports on infected colonies that never developed clinical disease symptoms visible to the apiculturist (8, 13) must be reevaluated. In a colony infected by a fast-killing genotype, only sporadic cells contain the ropy stage and foulbrood scale. Since these are the visible clinical symptoms of AFB, such an infection can be overlooked or remain unrecognized for a long time. Even though the classical clinical symptoms may not be apparent in such an infected colony, the colony nevertheless should be considered clinically infected since larvae are dying from the disease already.

The existence of more- or less-virulent strains of *P. larvae* subsp. *larvae*, as demonstrated in our study, is likely to influ-

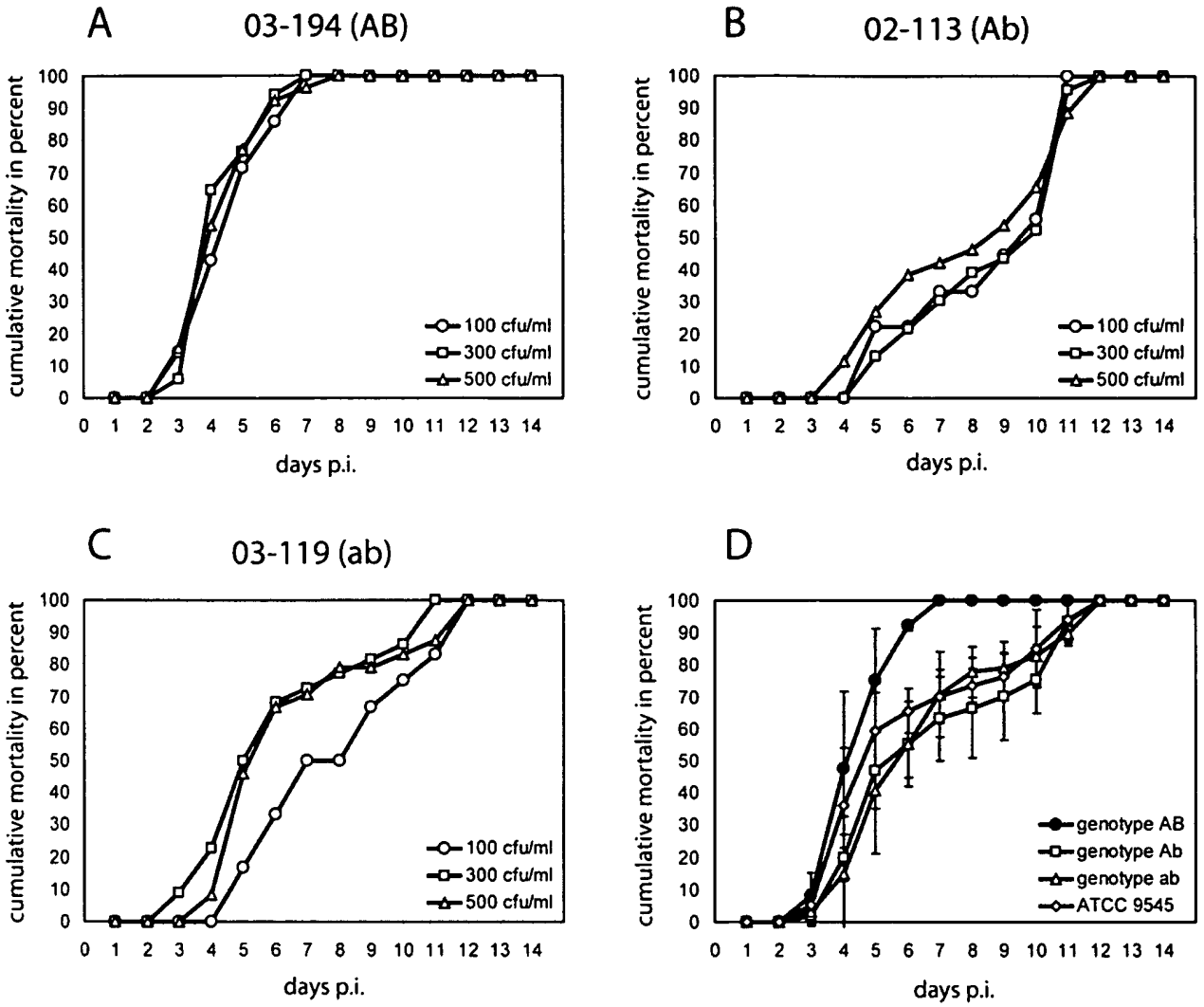

FIG. 2. Time course of infection. (A to C) Cumulative mortality was calculated per day postinfection (p.i.) as described in the text and expressed as percentage of the infected hosts (i.e., all larvae that died from AFB in this replicate). Concentrations tested are given in each panel. Results from three representative experiments are shown for each strain. (D) Cumulative mortality was calculated for each genotype from all strains as described in the text, using the concentration closest to the estimated LC_{50}. Therefrom, the mean cumulative proportion of AFB-dead larvae per day p.i. ±standard deviation was calculated for each genotype.

ence disease transmission and therefore to have important epidemiological consequences. *P. larvae* subsp. *larvae* is believed to be transmitted primarily through robbing of diseased colonies (horizontal transmission) (8). The mode of pathogen transmission (horizontal versus vertical) is an important factor determining virulence (11). In honeybees, which reproduce at colony level by colony fission, most pathogens are transmitted primarily vertically and, thus, are rather benign at colony level, since only rather strong colonies swarm. AFB infections are the exception to this rule, as disease transmission is actually favored by colony collapse (6). The scenario described here, with a suggested variation in virulence between genotypes at colony level (high virulence at larval level producing lower virulence at colony level), allows for selection among strains of *P. larvae* subsp. *larvae* that are more or less dependent on horizontal or vertical transmission, respectively. If all colonies that show clinical symptoms of disease are killed (as required by legislation in many countries), a selection pressure is probably imposed, selecting for less-virulent pathogen strains being primarily vertically transmitted, similarly to other honeybee pathogens (6). Recent evolutionary considerations suggest that interventions influencing disease frequency in a population, in

TABLE 2. *P. larvae* subsp. *larvae* genotype-specific LT_{100} and mortality after cell capping

P. larvae subsp. *larvae* genotype	LT_{100} (days p.i. [mean ± SD])	% Mortality after cell capping (mean ± SD)[a]
AB	7.8 ± 1.7	5.4 ± 3.2
Ab	11.2 ± 0.8	26.6 ± 7.3
ab	11.6 ± 0.6	20.2 ± 6.3
aβ	11.3 ± 0.8	26.3 ± 2.8

[a] One hundred percent mortality is the total number of larvae that died from AFB.

particular, interventions that influence mode of pathogen transmission, have the potential to tip the competitive balance in favor of less-virulent pathogen strains (5). This perspective of virulence management of pathogens in honeybee pathogens needs to be evaluated in the field.

This work was supported by grants from the Ministries for Agriculture from Brandenburg and Sachsen-Anhalt, Germany.

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APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2002, p. 3655–3660 Vol. 68, No. 7
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.7.3655–3660.2002

Differentiation of *Paenibacillus larvae* subsp. *larvae*, the Cause of American Foulbrood of Honeybees, by Using PCR and Restriction Fragment Analysis of Genes Encoding 16S rRNA

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Received 13 December 2001/Accepted 17 April 2002

A rapid procedure for the identification of *Paenibacillus larvae* subsp. *larvae*, the causal agent of American foulbrood (AFB) disease of honeybees (*Apis mellifera* L.), based on PCR and restriction fragment analysis of the 16S rRNA genes (rDNA) is described. Eighty-six bacterial strains belonging to 39 species of the genera *Paenibacillus*, *Bacillus*, *Brevibacillus*, and *Virgibacillus* were characterized. Amplified rDNA was digested with seven restriction endonucleases. The combined data from restriction analysis enabled us to distinguish 35 profiles. Cluster analysis revealed that *P. larvae* subsp. *larvae* and *Paenibacillus larvae* subsp. *pulvifaciens* formed a group with about 90% similarity; however, the *P. larvae* subsp. *larvae* restriction fragment length polymorphism pattern produced by endonuclease *Hae*III was found to be unique and distinguishable among other closely related bacteria. This pattern was associated with DNA extracted directly from honeybee brood samples showing positive AFB clinical signs that yielded the restriction profile characteristic of *P. larvae* subsp. *larvae*, while no amplification product was obtained from healthy larvae. The method described here is particularly useful because of the short time required to carry it out and because it allows the differentiation of *P. larvae* subsp. *larvae*-infected larvae from all other species found in apiarian sources.

American foulbrood (AFB) disease caused by the spore-forming bacterium *Paenibacillus larvae* subsp. *larvae* (15) (formerly *Bacillus larvae*) is a highly contagious, cosmopolitan disease of bacterial origin affecting the larval and pupal stages of honeybees (*Apis mellifera* L.). Infected individuals turn brown and then black, and the resultant mass becomes a hard scale of material deposited on the side of the cell. AFB is one of the few bee diseases capable of killing a colony, and it presents unique problems for prevention and control because the spores can remain viable for long periods and survive under adverse environmental conditions (17, 17a, 17b). The disease spreads when spores are carried on drifting bees, hive parts, beekeepers' clothes, and contaminated pollen or honey.

Govan et al. (13) and Dobbelaere et al. (8) reported the use of PCR for rapid identification of *P. larvae* subsp. *larvae* by using primers derived from gene regions encoding 16S rRNA (rDNA). Specific primers designed by Govan et al. (13) produced a single amplicon, whereas those designed by Dobbelaere et al. (8) produced four amplicons. The results of their analysis of a limited number of species from apiarian sources did not allow them to differentiate *P. larvae* subsp. *larvae* from *Paenibacillus larvae* subsp. *pulvifaciens*, the cause of powdery scale disease (15, 16), because both subspecies showed the same pattern. Dobbelaere et al. (8) concluded that the high degree of similarity between 16S rRNA genes of the two subspecies, about 99.44%, does not permit the design of specific primers for either of the two subspecies.

In addition, several *Paenibacillus* species and species of the genera *Bacillus*, *Brevibacillus*, and *Virgibacillus* were consistently reported as being isolated from apiarian sources (2, 9, 10, 11, 12). The complex microbial community of sporeformers includes *Paenibacillus alvei*, *Brevibacillus laterosporus*, and *Paenibacillus apiarius*, which are considered secondary bacterial invaders of larvae infected with European foulbrood (EFB), and also *P. larvae* subsp. *pulvifaciens* and *Bacillus coagulans*, which cause diseases of minor economical impact (1, 5, 16, 19, 20). Nevertheless, these bacteria can easily contaminate and overgrow plates of the slow-growing fastidious *P. larvae* subsp. *larvae*, making the correct diagnosis of AFB difficult unless selective media are used (1, 2).

The aim of this study was to assess the feasibility of using restriction fragment length polymorphism analysis (RFLP) of PCR-amplified 16S rDNAs for the differentiation of *P. larvae* subsp. *larvae* from other *Paenibacillus* organisms and from other spore-forming bacteria from apiarian sources and to assess its applicability to the direct and rapid diagnosis of AFB.

Strains and media. Eighty-six bacterial strains from diverse origins used in this study are listed in Table 1. For the isolation of *P. larvae* subsp. *larvae* strains from brood combs affected by AFB and from honey samples, previously described techniques were employed (2, 3). *Brevibacillus laterosporus* BLA 168 was isolated from honeybee larvae exhibiting symptoms of EFB, and Argentinian strains of *P. alvei*, *Bacillus cereus*, *Bacillus mycoides*, and *Bacillus megaterium* were recovered from honey as reported before (2).

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TABLE 1. Restriction patterns of PCR-amplified 16S rDNA genes among *Paenibacillus*, *Bacillus*, *Brevibacillus*, and *Virgibacillus* species and origins of the strains used in this study

Species, strain designation, and geographical origin[l]	Pattern obtained with restriction enzyme:						
	*Alu*I	*Msp*I	*Hae*III	*Hin*fI	*Cfo*I	*Rsa*I	*Taq*I
Paenibacillus larvae subsp. *larvae*							
ATCC 2574, United States	B	B	A	NRS[m]	A	A	A
PL113, PL225, Argentina[a]	B	B	A	NRS	A	A	A
PL295, PL296, United States[a]	B	B	A	NRS	A	A	A
PL201, PL203, Italy[a]	B	B	A	NRS	A	A	A
PL212, PL213, Canada[a]	B	B	A	NRS	A	A	A
PL228, PL230, France[a]	B	B	A	NRS	A	A	A
PL252, PL254, Spain[b]	B	B	A	NRS	A	A	A
PL284, PL286, Uruguay[c]	B	B	A	NRS	A	A	A
PL289, PL290, Japan[d]	B	B	A	NRS	A	A	A
PL56, PL57, Sweden[e]	B	B	A	NRS	A	A	A
PL29, PL31, New Zealand[f]	B	B	A	NRS	A	A	A
PL90, PL91, Germany[g]	B	B	A	NRS	A	A	A
PL68, PL70, Poland[h]	B	B	A	NRS	A	A	A
PL75 (CCM4483), PL76 (CCM4485), Czech Republic	B	B	A	NRS	A	A	A
PL301, PL302, UK[i]	B	B	A	NRS	A	A	A
PL100, Tunisia[a]							
PL304, PL305, Belgium[j]							
Paenibacillus larvae subsp. *pulvifaciens*							
CCM 38 (CCM), Czech Republic	B	B	B	B	A	A	A
NRRL B-3688, NRRL B-3670	B	B	B	B	A	A	A
NRRL B-14154, NRRL B-14152	B	B	B	B	A	A	A
ATCC 13537	B	B	B	B	A	A	A
SAG 4689-3, SAG 4689-6, United States[b]	B	B	B	B	A	A	A
Paenibacillus lentimorbus NRRL B-2522	F	D	NRS	F	A	A	A
Paenibacillus macquariensis NRRL B-14306	B	D	D	D	A	C	A
Paenibacillus glucanolyticus NRRL B-14679	B	E	G	E	A	E	D
Paenibacillus peoriae NRRL B-14750	B	E	H	G	A	A	A
Paenibacillus curdlanolyticus NRRL B-23243	J	E	NRS	K	E	D	B
Paenibacillus kobensis NRRL B-23299	D	J	I	B	G	B	E
Paenibacillus dendritiformis NRRL B-666	N	E	E	C	A	B	C
Paenibacillus lautus NRRL NRS-1000	B	E	G	D	A	A	A
Paenibacillus validus NRRL NRS-1347	N	E	E	C	A	B	C
Paenibacillus alginolyticus NRRL NRS-1351	H	D	J	J	D	C	B
Paenibacillus chondroitinus NRRL NRS-1356	B	D	D	H	A	B	A
Paenibacillus illinoisensis	B	D	H	D	A	A	A
Paenibacillus alvei							
NRRL B-383	I	E	NRS	H	A	A	A
m437a, m361, Argentina[a]	I	E	NRS	H	A	A	A
Paenibacillus amylolyticus NRRL B142	E	E	F	C	A	A	C
Paenibacillus apiarius ATCC 29575	B	E	NRS		A	C	A
Paenibacillus macerans NRRL NRS-924	A	H	C	B	B	D	B
Paenibacillus pabuli NRRL B-510	B	F	H	D	A	F	A
Paenibacillus polymyxa NRRL B_510	A	F	D	B	A	D	B
Paenibacillus azotofixans NRRL B-142	G	F	H	G	A	A	A
Paenibacillus chibensis ATCC 11377	B	E	L	G	A	A	A
Paenibacillus thiaminolyticus	A	D	C	C	B	D	B
Paenibacillus popilliae ATCC 14706	B	D	NRS	C	C	A	A
Paenibacillus borealis KK19, Finland[k]	B	D	D	D	A	C	A
Bacillus azotoformans NRRL B-14310	A	I	O	L	B	D	B
Bacillus circulans ATCC 4515	C	C	C	C	A	B	B
Bacillus cereus							
ATCC 11778	D	E	E	C	A	B	C
m432, m436, Argentina[a]	D	E	E	C	A	B	C
Bacillus coagulans ATCC 35670	A	D	C	B	B	D	B
Bacillus licheniformis NRRL B-1001	D	D	C	B	A	D	B
Bacillus megaterium							
NRRL B-939	A	E	C	C	A	B	C
m412, m440a, Argentina[a]	A	E	C	C	A	B	C
Bacillus mycoides							
ATCC 10206	K	E	K	C	A	B	C
m425, m440b, Argentina[a]	K	E	K	C	A	B	C
Bacillus thuringiensis ATCC 10792	D	E	E	C	A	B	C
Bacillus pumilus ATCC 7061	A	D	C	B	B	D	B

Continued on following page

TABLE 1—*Continued*

Species, strain designation, and geographical origin[l]	Pattern obtained with restriction enzyme:						
	*Alu*I	*Msp*I	*Hae*III	*Hin*fI	*Cfo*I	*Rsa*I	*Taq*I
Bacillus subtilis ATCC 10783	A	D	C	B	B	D	B
Bacillus sphaericus ATCC 245	A	E	C	C	A	D	C
Bacillus firmus ATCC 8247	G	F	D	C	A	A	C
Virgibacillus pantothenticus ATCC 14567	L	E	M	B	F	G	C
Brevibacillus laterosporus							
CCT 31 (CCT)	M	G	N	C	A	B	B
BLA 168, Argentina[a]	M	G	N	C	A	B	B

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[l] ATCC, American Type Culture Collection, Rockville, Md.; CCM, Czech Collection of Microorganisms, Brno, Czech Republic; CCT, Coleçao de Culturas Tropical, Fundaçao André Tosello, Campinas, SP, Brazil; NRRL, Northern Regional Research Laboratory, Peoria, Ill.
[m] NRS, no recognition site.

P. larvae subsp. *larvae*, *P. larvae* subsp. *pulvifaciens*, *P. amylolyticus*, *P. lautus*, *P. illinoisensis*, and *P. chibensis* strains were grown on MYPGP agar (6) at 37°C for 48 h; the other *Paenibacillus* species were grown on MYPGP agar at 30°C for 24 to 48 h, except *P. macquariensis*, which was grown at 22°C, and *P. dendritiformis*, which was grown on Luria-Bertani agar at 37°C. *Bacillus*, *Brevibacillus*, and *Virgibacillus* species were grown on tryptic soy agar at 30°C for 24 h, with the exception of *B. coagulans*, which was incubated at 37°C. Purity was confirmed by colony morphology and microscopic examination of bacterial smears.

DNA preparation. Bacterial cells for DNA extraction were grown at the appropriate temperature and medium under aerobic conditions for 24 to 48 h according to the species used. For bacterial DNA preparation, a rapid procedure using whole cells from plates was employed (3). After centrifugation to remove bacterial debris and resin, the supernatant was used as the DNA template.

PCR amplification and RFLP analysis of 16S rDNA. Primers U1 and U2 described by Ash et al. were used for PCR amplification of 16S rRNA genes from *Bacillus*, *Paenibacillus*, *Brevibacillus*, and *Virgibacillus* species (4). These primers were derived from conserved regions and capable of amplifying about 1.1 kb of 16S rDNA from *Bacillus* species and closely related genera. The PCR mixtures, which contained 1.5 μl of deoxynucleotide mixture (2 mM each), 1.25 μl of a mixture of both primers (10 mM each), 1.5 μl of Promega (Buenos Aires, Argentina) reaction buffer, 1.0 μl of $MgCl_2$ (25 μM), 5 μl of supernatant DNA, and sterile deionized water to bring the final volume to 25 μl, were pretreated at 94°C before 1 U of *Taq* polymerase (Promega Corp.) was added. PCR amplification was carried out according to the protocol of Ash et al. (4). PCR products were examined by using agarose (0.8%) gel electrophoresis and visualized by using ethidium bromide and UV light.

After amplification, subsamples of about 5 μl were incubated overnight with endonucleases *Rsa*I, *Hae*III, *Msp*I, *Alu*I, *Hin*fI, *Taq*I, and *Cfo*I (Promega) according to the manufacturer's specifications. RFLP analysis was performed by electrophoresis in a 2% agarose gel at 80 V for 2.30 h.

We found that species belonging to the genera *Paenibacillus*, *Bacillus*, *Brevibacillus*, and *Virgibacillus* consistently yielded a PCR amplification product of about 1,100 bp. In our analysis of the 16S rRNA gene, we assayed the variation at seven restriction sites that were thought to provide an RFLP pattern diagnostic of *P. larvae* subsp. *larvae*. It was found that analysis of 86 strains from different sources and classified as belonging to 39 species allowed us to place them in 35 composite profiles following digestion with *Rsa*I, *Hae*III, *Msp*I, *Alu*I, *Hin*fI, *Taq*I, and *Cfo*I by using the "combined gels" option of Gelcompare. The program FreeTree (14) was used for the construction of a phylogenetic tree (Fig. 1B) and for jackknife analysis by using a binary matrix based on RFLP characters (Nei-Li distances; neighbor-joining tree-construction method; 1,000 resampled data sets).

The result of the analysis shown in Fig. 1 revealed that *P. larvae* subsp. *larvae* and *P. larvae* subsp. *pulvifaciens* formed a group with about 90% similarity. However, the *Hae*III restriction pattern of *P. larvae* subsp. *larvae* was found to be unique and allowed us to distinguish it from other closely related bacteria. Indeed, none of eight *P. larvae* subsp. *pulvifaciens* strains we examined showed the two *Hae*III fragments of about 300 and 470 bp, respectively, which were characteristic of *P. larvae* subsp. *larvae* strains ($n = 32$) (Fig. 2A). Further evidence that supported subspecies differentiation was obtained, extending this analysis to 365 isolates of *P. larvae* subsp. *larvae* from different origins that showed the same *Hae*III restriction pattern (data not shown); in addition, *Hin*fI restriction analysis, unlike with *P. larvae* subsp. *pulvifaciens*, revealed no recognition site in *P. larvae* subsp. *larvae* strains (Table 1). On the

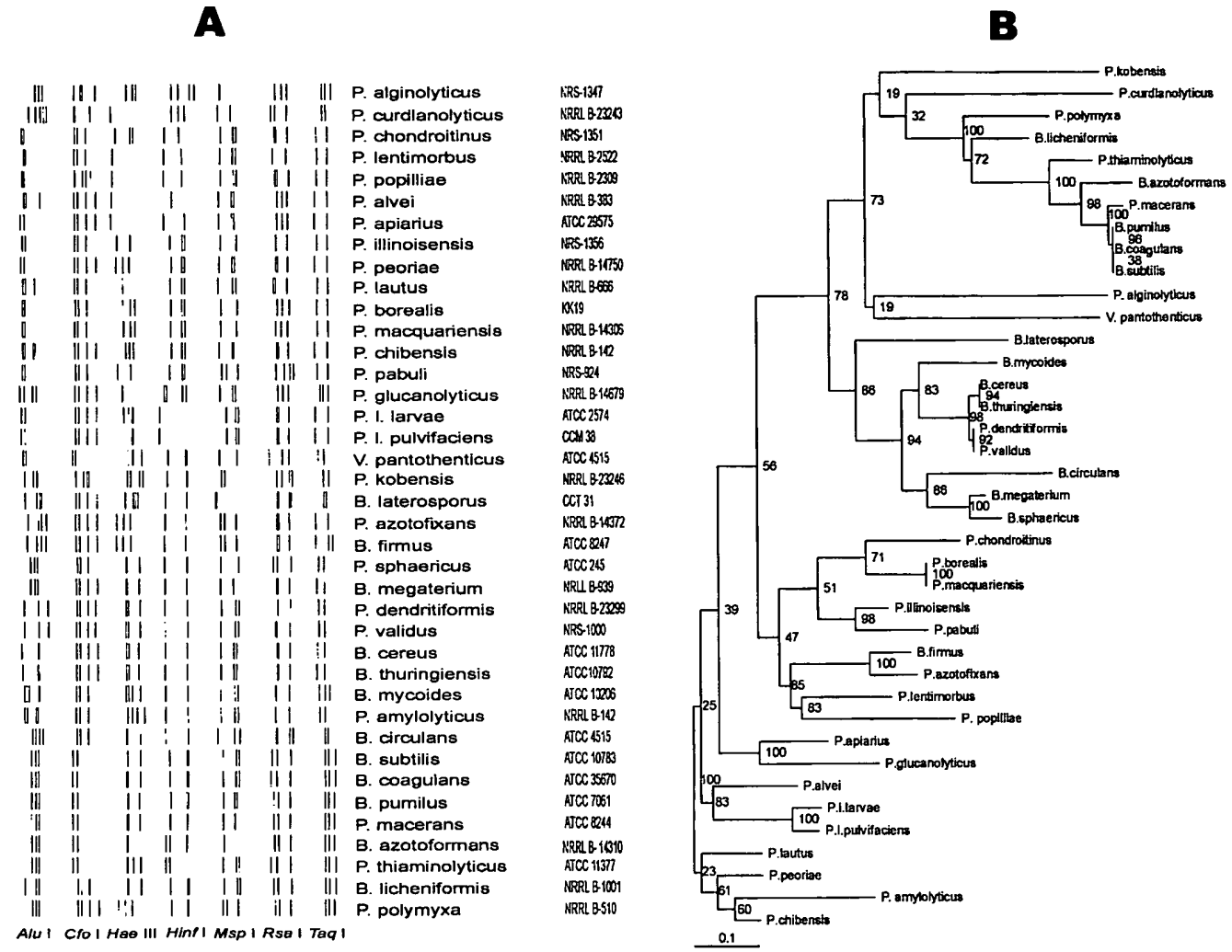

FIG. 1. (A) Combined restriction patterns of PCR-amplified 16S rDNA from representative species of the genera *Paenibacillus*, *Bacillus*, *Brevibacillus*, and *Virgibacillus* obtained by using the endonucleases *Alu*I, *Cfo*I, *Hae*III, *Hin*fI, *Msp*I, *Rsa*I, and *Taq*I. (B) Phylogenetic tree constructed on the basis of RFLP data by the neighbor-joining method using FreeTree software. Jackknife values are indicated at the branching points (1,000 replicates).

other hand, profiles obtained with *Alu*I, *Cfo*I, *Rsa*I, and *Taq*I were found to be identical in both subspecies. In addition, the *Msp*I restriction patterns shown by *P. larvae* subsp. *larvae* and subsp. *pulvifaciens* were identical, whereas they differed from that of other species we examined (Fig. 2B). This relatedness between *P. larvae* subsp. *larvae* and *P. larvae* subsp. *pulvifaciens* is in agreement with previous evidence obtained by using a polyphasic approach (15). Differences between pairs of restriction patterns, as found here, could be simply explained in terms of gain or loss of only one or two restriction sites, which indicates indeed that these two subspecies are genetically closely related.

In a few cases, pairs or groups (e.g., *B. cereus* and *B. thuringiensis*; *B. subtilis*, *B. coagulans*, and *B. pumilus*; and *P. borealis* and *P. macquariensis*) were not differentiated by the set of endonucleases that we used (Table 1; Fig. 1A). The use of other endonucleases or DNA sequencing may provide a basis for their differentiation.

We conclude that the 16S rRNA gene is polymorphic among the aerobic spore-forming bacterial species predominant in apiarian sources. However, intraspecies polymorphism was not detected among the 32 *P. larvae* subsp. *larvae* strains obtained from diverse geographic regions. More interesting, we found that restriction pattern analysis revealed a distinct genotype for *P. larvae* subsp. *larvae* which may be useful for its identification, since the use of the endonucleases *Msp*I, *Hin*fI, and *Hae*III would result in the recognition of *P. larvae* subsp. *larvae* among apiarian bacteria.

Direct detection in honeybee larva samples. Current procedures to detect AFB disease are based on direct field inspection of the hives and on the use of selective bacterial growth media combined with PCR methods (1, 2, 3, 13). Overall, they possess some limitations, since occasionally clinical symptoms are ambiguous and several days are required to reach a conclusive diagnosis. Therefore, in order to assess whether the 16S rDNA-RFLP analysis might be useful to reveal and confirm *P. larvae* subsp. *larvae* infection in hives, we carried out the following assays. Larvae exhibiting clinical symptoms of AFB

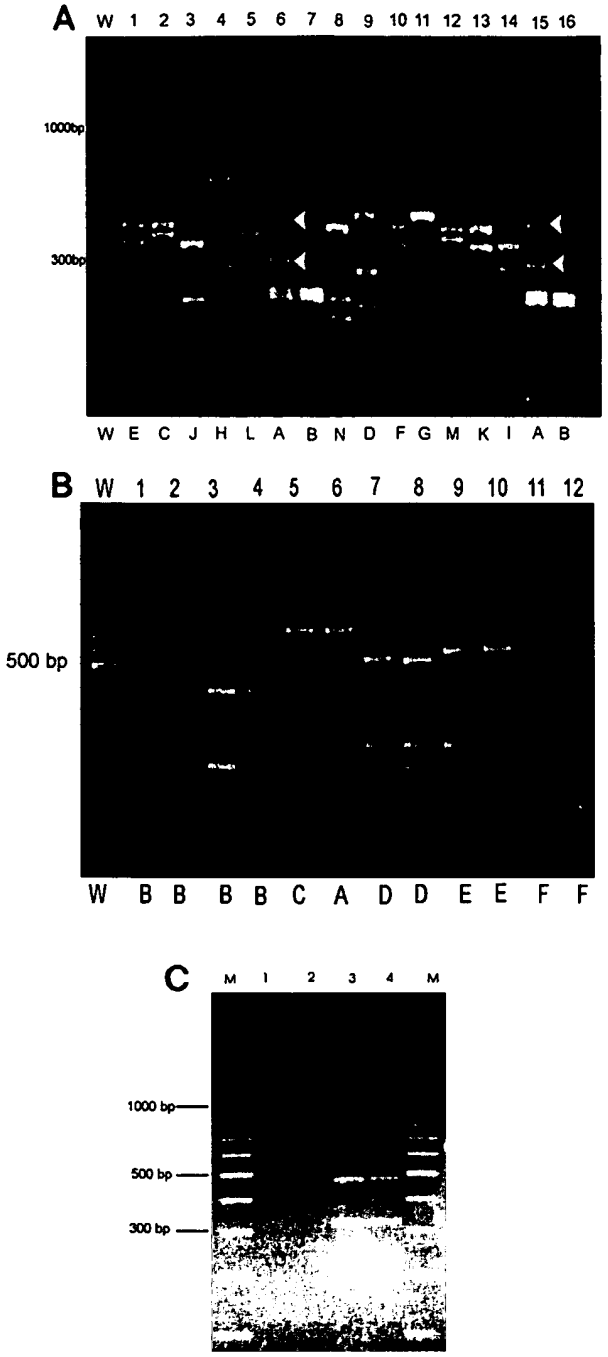

FIG. 2. (A) Gel electrophoresis of a PCR-amplified 16S rDNA fragment of 1,100 bp digested with *Hae*III. Similar restriction patterns were grouped and assigned the same letter (A to N). Lanes: W, molecular weight marker (100-bp ladder; Biodynamics, Buenos Aires, Argentina); 1, *B. cereus* ATCC 11778; 2, *Paenibacillus macerans* ATCC 8244; 3, *Paenibacillus alginolyticus* NRRL NRS-1347; 4, *Paenibacillus peoriae* NRRL B-14750; 5, *Paenibacillus chibensis* NRRL B-142; 6, *P. larvae* subsp. *larvae* ATCC 2574; 7, *P. larvae* subsp. *pulvifaciens* CCM 38; 8, *Brevibacillus laterosporus* CCT 31; 9, *Paenibacillus borealis* KK19; 10, *Paenibacillus amylolyticus* NRRL B 142; 11, *Paenibacillus glucanolyticus* NRRL B-14679; 12, *Virgibacillus pantothenticus* ATCC 14567; 13, *B. mycoides* (ATCC 10206); 14, *Paenibacillus kobensis* NRRL B-23246; 15, *P. larvae* subsp. *larvae* PI 113; 16, *P. larvae* subsp. *pulvifaciens* ATCC 13537. Fragments of about 300 and 470 bp, respectively, that distinguish *P. larvae* subsp. *larvae* (lanes 6 and 15) from *P. larvae* subsp. *pulvifaciens* (lanes 7 and 16) are indicated by arrowheads. (B) Gel electrophoresis of a PCR-amplified 16S rDNA fragment of 1,100 bp digested with *Msp*I. Similar restriction patterns were grouped and assigned the same letter (A to G). Lanes: W, molecular weight marker (100-bp ladder; Biodynamics); 1, *P. larvae* subsp. *larvae* ATCC 2574; 2, *P. larvae* subsp. *larvae* PI 113; 3, *P. larvae* subsp. *pulvifaciens* CCM 38; 4, *P. larvae* subsp. *pulvifaciens* SAG; 5, *Bacillus circulans* ATCC 4515; 6, *Bacillus firmus* ATCC 8247; 7, *Paenibacillus azotofixans* NRRL B-14372; 8, *Brevibacillus laterosporus* CCT 31; 9, *Bacillus subtilis* ATCC 10783; 10, *Paenibacillus macerans* ATCC 8244; 11, *P. alvei* NRRL B-383; and 12, *Bacillus thuringiensis* ATCC 10792. (C) Agarose gel showing PCR-RFLP results from healthy and diseased larvae. Lanes: M, molecular weight marker (100-bp ladder; Biodynamics); 1, PCR of AFB-diseased larvae using primers U1/U2; 2, PCR of healthy larvae; 3, PCR-amplified 16S rDNA fragment of 1,100 bp from *P. larvae* subsp. *larvae* ATCC 2574 digested with *Hae*III; 4, PCR-amplified 16S rDNA fragment from AFB-infected larvae digested with *Hae*III.

were removed from the cells by using a toothpick and thoroughly mixed with 1 ml of sterile distilled water (two larval remains or scales per tube). One hundred microliters of this mixture was diluted in 900 μl of sterile distilled water, vortex mixed, and centrifuged at 3,200 × *g* for 5 min. Fifty microliters of the supernatant was heated at 95°C for 15 min (8) and centrifuged at 3,200 × *g* for 5 min. Subsamples of the supernatant were used as DNA templates in the PCR amplification as described above. Similar treatment was applied to healthy larvae 2, 5, and 19 days old and also to larval remains infected with chalkbrood caused by the fungus *Ascosphaera apis* (18) and EFB caused by the bacterium *Melissococcus plutonius* (formerly *Melissococcus pluton*) (5, 7), which were assessed as controls. AFB, EFB, and chalkbrood were confirmed by using standard microscopic and microbiological techniques (1, 5, 7, 18, 19). The results in Fig. 2C reveal that DNA extracted from larva samples associated with AFB symptoms consistently amplified the 1,100-bp fragment which, after incubation with endonuclease *Hae*III, gave an RFLP pattern identical to that found to be characteristic of *P. larvae* subsp. *larvae*. No amplification was detected with extracts from healthy larvae, EFB-diseased larvae, or chalkbrood mummies (dried dead larvae affected by chalkbrood disease). Furthermore, by using larval samples carrying mixed spore-forming bacterial populations that had been described by Alippi (1), a unique *Hae*III restriction pattern identical to that of *P. larvae* subsp. *larvae* was observed (data not shown). We assume that the high level of *P. larvae* subsp. *larvae* spores present in larva samples may indicate that DNA from *P. larvae* subsp. *larvae* outcompetes those from other bacteria as a template in the PCR.

Finally, DNA fingerprint analysis using the primers BOX, REP, and ERIC revealed four different genotypes within the *P. larvae* subsp. *larvae* collection we examined, which were demonstrated to be genetically diverse even though the 16S rDNA-RFLP pattern was identical (3; Alippi et al., unpublished data).

Our study provides a method that appears to be helpful in distinguishing *P. larvae* subsp. *larvae* from other *Paenibacillus* organisms, particularly those that are closely related, such as *P. larvae* subsp. *pulvifaciens*, and also from the spore-forming species which are commonly found in samples from apiarian environments. Since this procedure allows the identification of *P. larvae* subsp. *larvae* obtained either from culture or from

larvae, we believe it can be applied to the reliable and rapid diagnosis of AFB (in about 4 h), in contrast to classical microbiological methods, which require at least 2 days.

This research was supported by a grant from ANPCyT, Argentina (BID 1201/OC-AR PICT 08-03857), and from the IFS, Sweden (A.M.A.). A.M.A. and O.M.A. are Career Investigators of CIC and CONICET, respectively, and A.C.L. is a recipient of a scholarship from ANPCyT, Argentina.

We thank the curator of the NRRL Collection, N. Nakamura, for supplying bacterial cultures, those (listed in Table 1) who provided AFB samples or bacterial isolates, and F. J. Reynaldi for collecting larval samples.

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Antagonistic interactions between honey bee bacterial symbionts and implications for disease

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Published: 21 March 2006

BMC Ecology 2006, **6**:4 doi:10.1186/1472-6785-6-4

Received: 16 November 2005

Accepted: 21 March 2006

This article is available from: http://www.biomedcentral.com/1472-6785/6/4

Abstract

Background: Honey bees, *Apis mellifera*, face many parasites and pathogens and consequently rely on a diverse set of individual and group-level defenses to prevent disease. One route by which honey bees and other insects might combat disease is through the shielding effects of their microbial symbionts. Bees carry a diverse assemblage of bacteria, very few of which appear to be pathogenic. Here we explore the inhibitory effects of these resident bacteria against the primary bacterial pathogen of honey bees, *Paenibacillus larvae*.

Results: Here we isolate, culture, and describe by 16S rRNA and protein-coding gene sequences 61 bacterial isolates from honey bee larvae, reflecting a total of 43 distinct bacterial taxa. We culture these bacteria alongside the primary larval pathogen of honey bees, *Paenibacillus larvae*, and show that many of these isolates severely inhibit the growth of this pathogen. Accordingly, symbiotic bacteria including those described here are plausible natural antagonists toward this widespread pathogen.

Conclusion: The results suggest a tradeoff in social insect colonies between the maintenance of potentially beneficial bacterial symbionts and deterrence at the individual and colony level of pathogenic species. They also provide a novel mechanism for recently described social components behind disease resistance in insect colonies, and point toward a potential control strategy for an important bee disease.

Background

Insects, like many eukaryotes, can be strongly affected by the microbes they harbor. Bacterial associates of insects are implicated in the degradation of plant materials and other foods, regulation of pH, synthesis of vitamins and, in relatively rare cases, induction of disease [1]. Insect-bacteria associations range from facultative short-term interactions to highly codependent symbioses [2]. Social insects provide unique resources for microbial symbionts, thanks to the high density of individuals within colonies, sharing of food and other resources, and the coexistence of colony members from multiple generations. Not surprisingly then, symbioses between social insect species and microbial species are common and often highly coevolved. Many species of termites and ants, for example, are obligately tied to specific microbes for their nutritional needs [3-6].

Honey bees, *Apis mellifera*, support a diverse microbial biome [7]. Recent surveys from adult bees indicate the presence of dozens of bacterial taxa, ranging from gram-positive bacteria to alpha-, beta-, and gamma-proteobac-

teria [7,8]. While a few of these bacterial species are clearly pathogenic, most have never been associated with honey bee disease and their impacts on each other and their honey bee hosts are unknown.

Now is an ideal time to explore the diversity of insect endosymbiotic bacteria. First, an immense library of sequence data for 16S rRNA loci and other robust markers allows the precise identification of many associated species, even those that resist cultivation [9]. Over 200,000 bacterial entries exist currently for 16S rRNA, and 16S sequences can place most surveyed bacterial taxa securely into genera, if not species. Additionally, genomic studies of diverse bacterial species allow new insights into the mechanisms of maintenance and growth for these microbes as well as their potential impacts on the health of their insect hosts [10-12]. Finally, recent community-level surveys of bacterial diversity across different arthropods [13-18] allow a comparative approach toward understanding roles played by bacteria during different host life stages, and in different organs of the body.

Recently, we described four honey bee bacterial symbionts with clear bacteriostatic effects against the most virulent and widespread honey bee pathogen, the gram-positive bacterium *Paenibacillus larvae larvae* [19]. Here, we present a more complete survey of bacterial species isolated from honey bee larvae: the life stage at which they are most susceptible to invasion by pathogenic bacteria. We used 16S universal bacterial primers to identify bacteria and *in vitro* inhibition assays to quantify the abilities of each of these isolates to inhibit *P. l. larvae*. We present wide-ranging taxa capable of inhibiting this pathogen and show considerable variation within and across colonies in the distribution of inhibitory taxa. The results have general implications for the expression of bacterial virulence in insects and for the maintenance of both beneficial and disease-associated bacteria in social insects. They also point to new avenues for the prophylactic or therapeutic treatment of honey bee diseases.

Results and discussion

Species distribution

Sequenced isolates (n = 61) were placed by 16S rRNA sequence similarity into four bacterial genera: *Acinetobacter, Bacillus, Brevibacillus,* and *Stenotrophomonas*. A total of 43 16S haplotypes representing a minimum of 15 bacterial taxa were identified from the survey (Table 1, Fig. 2). Most isolates fell within the genus *Bacillus* (n = 41). Of these, 29 belonged to the *B. cereus* group and differed only slightly at 16S. While 16S rRNA fails to confidently resolve this species group, 26 showed best matches to *B. cereus s.s. isolates*, while three were closest to *B. thuringiensis*. Additional resolution of this group was provided by GLyP and PyC sequencing for a subset of isolates. These sequences reflected a broad range of haplotypes with matches to members on each extreme of the *cereus* group (Fig. 3). Outside of the *cereus* group, the remaining *Bacillus* spp. matched *B. fusiformis, B. flexus, B. mycoides,* and *B. niabensis* (Table 1). Three additional *Bacillus* isolates showed <97% sequences similarity to deposited sequences in GenBank and as such were not reliably assignable to species. However two of those three isolates fell in a clade with *B. fusiformis* and the last isolate extended off the *B. cereus* clade (Fig 2). 10 isolates were indistinguishable from *Stenotrophomonas maltophilia* (Table 1). Although all three isolates of *Acinetobacter* fell into one clade in the 16S tree, BLASTN only matched one of the three isolates to a species while the remaining were matched solely to a genus. Seven isolates were placed into the genus *Brevibacillus*, with close matches to *Br. formosus* (n = 4), *Br. centrosporus* (n = 1), and *Br. brevis* (n = 1). The final *Brevibacillus* isolate did not match particularly closely to a taxon in the 16S database. None of the genera represented in this survey matched genera found in a previous 16S survey of bacteria from adult honey bees [8], suggesting that bacterial sequencing in bees will continue to identify novel taxa.

Diversity of inhibitory species

Inhibition zones, when present, ranged from a <10 mM radius around the isolated bacteria to complete inhibition of *P. larvae* across the dish. A total of 23 bacterial isolates consistently inhibited *P. larvae*. Isolates that inhibited *P. larvae* were evenly distributed across the sampled taxa (Fig. 2). Out of 43 *Bacillus sp.* isolates, ten showed consistent inhibition while seven additional isolates showed conditional inhibition. These seven all fell into the *Bacillus cereus* group. Of the ten *Bacillus* isolates with consistent inhibition, 8 were *B. cereus* and two were *B. fusiformis*. Thus, by the current *in vitro* assay, *B. niabensis, B. circulans, B. flexus,* and *B. mycoides* isolates were not inhibitory of *P. larvae*. All isolates that were matches with *Brevibacillus formosus* consistently inhibited the pathogen, while a single isolate placed with *B. centrosporus* did not inhibit. All isolates tied to *Stenotrophomonas maltophilia* consistently inhibited *P. larvae*, while two of three *Acinetobacter* isolates showed inhibition.

Distribution across individual bees and colonies

More bacteria and bacterial species were isolated, per individual, from 7-day-old larvae than from one-day larvae (28 of 55 larvae at 7 days, 28 of 306 larvae at 24 hr, G test, $p < 0.0001$; Table 2). Since larvae for both incubation lengths were collected at the same time from the colony, this difference does not reflect a greater chance for larvae to be inoculated in the nest as they age. Instead, the differences presumably reflect quantitatively higher bacterial loads in older individuals, such that these bacteria were more readily cultured. There was no apparent difference in species type or overall diversity between young and

Table 1: Isolate identification, 16SrRNA best match, similarity and BLASTN e-value, and radial zone of inhibition against the bee pathogen *Paenibacillus l. larvae*.

MB ID	Genbank ID	Best Match	% Similarity	e-value	Inhib.
BRL02-1	DQ339635	*Brevibacillus formosus*	98	0	23
BRL02-2	DQ339636	*Brevibacillus formosus*	98	0	13
BRL02-3A	DQ339637	*Brevibacillus brevis*	97	0	9
BRL02-3B	DQ339638	*Brevibacillus formosus*	98	0	>40
BRL02-4	DQ339639	*Stenotrophomonas maltophilia*	99	0	0
BRL02-5	DQ339640	*Stenotrophomonas maltophilia*	88	0	0
BRL02-6A	DQ339641	*Bacillus fusiformis*	98	0	>40
BRL02-6B	DQ339642	*Stenotrophomonas maltophilia*	99	0	>40
BRL02-7	DQ339643	*Stenotrophomonas maltophilia*	97	0	0
BRL02-8	DQ339644	*Stenotrophomonas maltophilia*	99	0	>40
BRL02-9	DQ339645	*Stenotrophomonas maltophilia*	98	0	>40
BRL02-11	DQ339646	*Stenotrophomonas maltophilia*	88	-179	0
BRL02-13	DQ339647	*Bacillus niabensis*	99	0	0
BRL02-14	DQ339648	*Bacillus cereus*	99	0	0
BRL02-16	DQ339649	*Bacillus cereus*	98	0	0
BRL02-17	DQ339650	*Bacillus cereus*	99	0	0
BRL02-19	DQ339651	*Stenotrophomonas maltophilia*	99	0	18
BRL02-20	DQ339652	*Stenotrophomonas maltophilia*	99	0	35
BRL02-21	DQ339653	*Bacillus cereus*	99	0	0
BRL02-22	DQ339654	*Bacillus cereus*	99	0	0
BRL02-23	DQ339655	*Bacillus cereus*	99	0	0
BRL02-24	DQ339656	*Brevibacillus centrosporus*	79	2E-90	0
BRL02-25	DQ339657	*Bacillus cereus*	98	0	0
BRL02-26	DQ339658	*Bacillus cereus*	99	0	0
BRL02-27	DQ339659	*Bacillus cereus*	100	0	A
BRL02-28	DQ339660	*Bacillus cereus*	99	0	A
BRL02-29	DQ339661	*Bacillus cereus*	99	0	0
BRL02-30	DQ339662	*Bacillus cereus*	91	0	0
BRL02-31	DQ339663	*Bacillus cereus*	99	0	12
BRL02-32	DQ339664	*Bacillus cereus*	99	0	0
BRL02-33	DQ339665	*Bacillus cereus*	98	0	0
BRL02-34	DQ339666	*Bacillus fusiformis*	99	0	0
BRL02-37	DQ339667	*Bacillus fusiformis*	98	0	>40
BRL02-38	DQ339668	*Stenotrophomonas maltophilia*	99	0	>40
BRL02-39	DQ339669	*Bacillus cereus*	99	0	0
BRL02-40	DQ339670	*Bacillus cereus*	99	0	0
BRL02-41	DQ339671	*Bacillus cereus*	99	0	0
BRL02-42A	DQ339672	*Acinetobacter calcoaceticus*	99	0	>40
BRL02-42B	DQ339673	*Bacillus cereus*	99	0	>40
BRL02-43	DQ339674	*Bacillus cereus*	98	0	0
BRL02-44	DQ339675	*Bacillus cereus*	99	0	0
BRL02-45	DQ339676	*Bacillus fusiformis*	97	0	>40
BRL02-46	DQ339677	*Brevibacillus centrosporus*	97	0	0
BRL02-52	DQ339678	*Bacillus mycoides*	98	0	0
BRL02-54	DQ339679	*Bacillus cereus*	99	0	0
BRL02-55	DQ339680	*Bacillus cereus*	99	0	0
BRL02-56	DQ339681	*Acinetobacter sp.*	100	0	0
BRL02-57	DQ339682	*Bacillus cereus*	100	0	0
BRL02-60	DQ339683	*Acinetobacter sp.*	100	0	>40
BRL02-61	DQ339684	*Bacillus cereus*	98	0	0
BRL02-62	DQ339685	*Bacillus cereus*	99	0	0
BRL02-64	DQ339686	*Bacillus circulans*	96	0	0
BRL02-65	DQ339687	*Bacillus flexus*	99	0	0
BRL02-66	DQ339688	*Bacillus flexus*	99	0	0
BRL02-67	DQ339689	*Bacillus flexus*	99	0	0
BRL02-68	DQ339690	*Bacillus cereus*	100	0	0
BRL02-69	DQ339691	*Bacillus flexus*	100	0	0
BRL02-70	DQ339692	*Bacillus cereus*	99	0	18
BRL02-71	DQ339693	*Bacillus cereus*	100	0	0
BRL02-76	DQ339694	***Bacillus cereus***	**97**	**0**	**8**

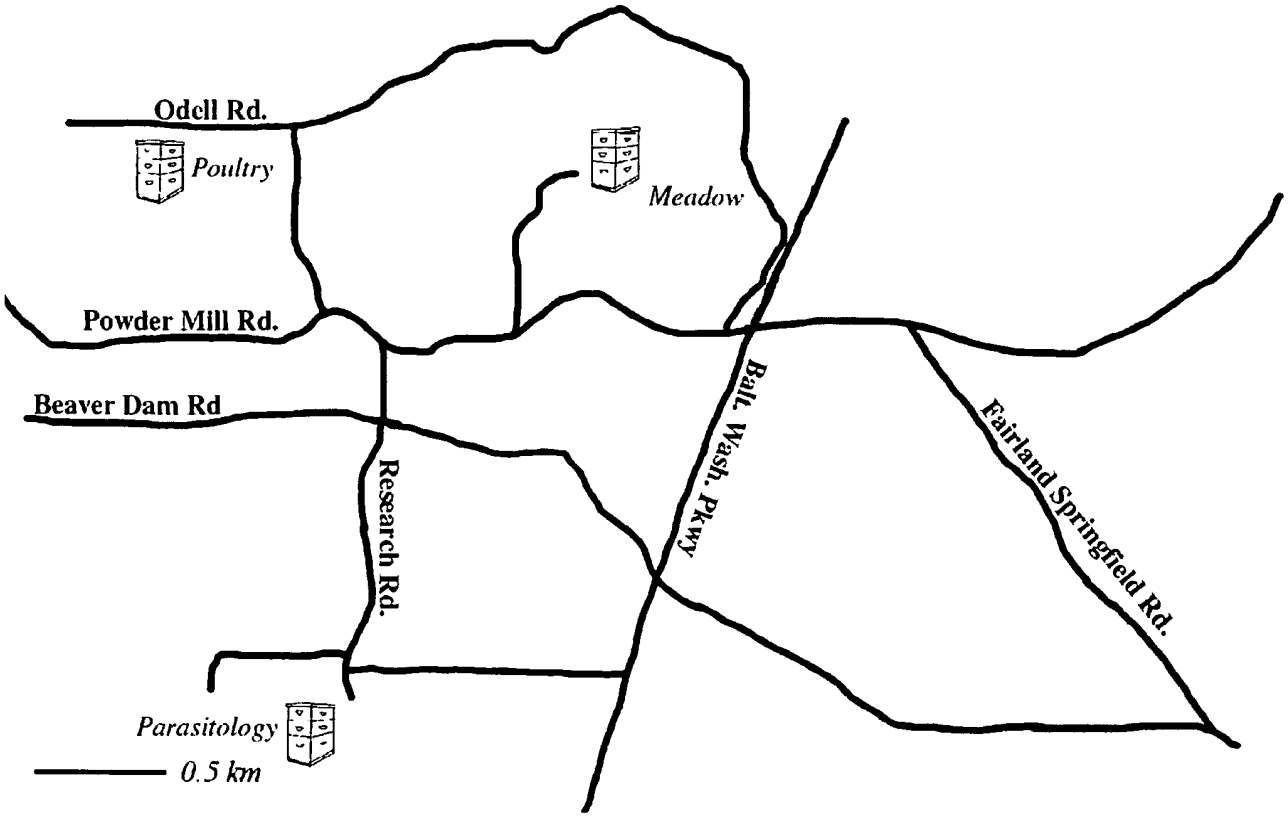

Figure 1
Apiary Map. Map of the apiaries in which honey bee larvae were collected. Q3, Q7, and LID larvae resided in colonies within the Meadow apiary. Size bar represents 0.5 km.

older larvae, nor was there a significant difference in the likelihood that a collected bacterial species was inhibitory toward *P. larvae* (likelihood ratio, $p = 0.2$).

There was a significant difference across sites in the frequency of resident, cultivable, bacterial species. Two sites had very low bacterial levels. One of these sites had been established shortly before collections took place. In this site (LID), only 4 out of 136 larvae (from 4 out of 34 colonies) showed bacterial growth. A second site, (Poultry) showed similarly low rates of cultivable bacteria (2.2%, n = 45 larvae). By contrast, measurable bacterial loads were present in 13% (n = 63) and 24% (n = 46) of larvae from the Parasitology and Meadow sites, respectively. Within sites, there was significant variation across colonies in the tendency of their larvae to harbor bacteria (nested ANOVA, $p < 0.001$), and in the specific bacteria found in certain colonies. More widespread surveys will be useful for defining colony-level bacterial 'signatures', an important step in determining the impacts of symbiotic bacteria on colony health.

Conclusion

We describe wide-ranging endogenous bacterial taxa that are capable of inhibiting an important honey bee pathogen and show considerable variation within and across colonies in the distribution of these taxa. The results have general implications for the expression of bacterial virulence in insects and for the maintenance of both beneficial and disease-associated bacteria in social insects. They also point to new avenues for the prophylactic or therapeutic treatment of honey bee diseases. None of the genera represented in this survey matched genera found in a previous 16S survey of bacteria from adult honey bees [8], although they do mimic, broadly, the microbial biome measured in bee colonies to date (as reviewed by Gilliam, [7]).

Most of the bacteria cultivated in this study belonged to the genus *Bacillus*, a result that is consistent with the high frequency of isolates placed in this genus by Gilliam and colleagues [7]. Among the *Bacillus* species, the majority fell into the *Bacillus cereus* group. Both 16S rRNA sequenc-

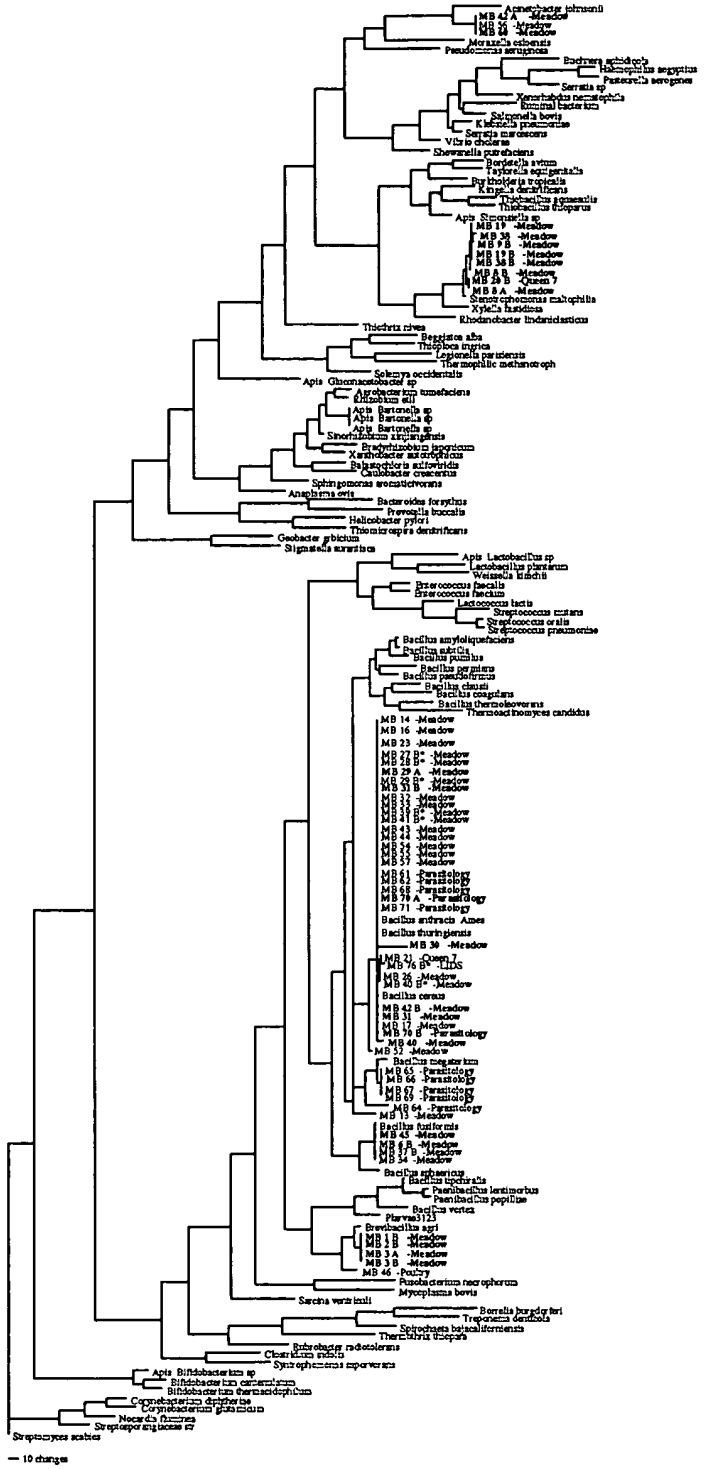

Figure 2

16S rRNA parsimony tree. Maximum parsimony tree showing relationships between bacterial isolates from this study, other bacteria known from bees, and representative members of the major bacterial clades. Tree based on an alignment of 850 nt from the 5' end of the 16S rRNA gene. Bacteria from this study shown as MB##, followed by site of collection. Isolates that showed inhibition of the honey bee pathogen *P. larvae* shown in bold type. Names in bold represent those isolates that inhibited and isolates followed by an asterick did not inhibit consistently.

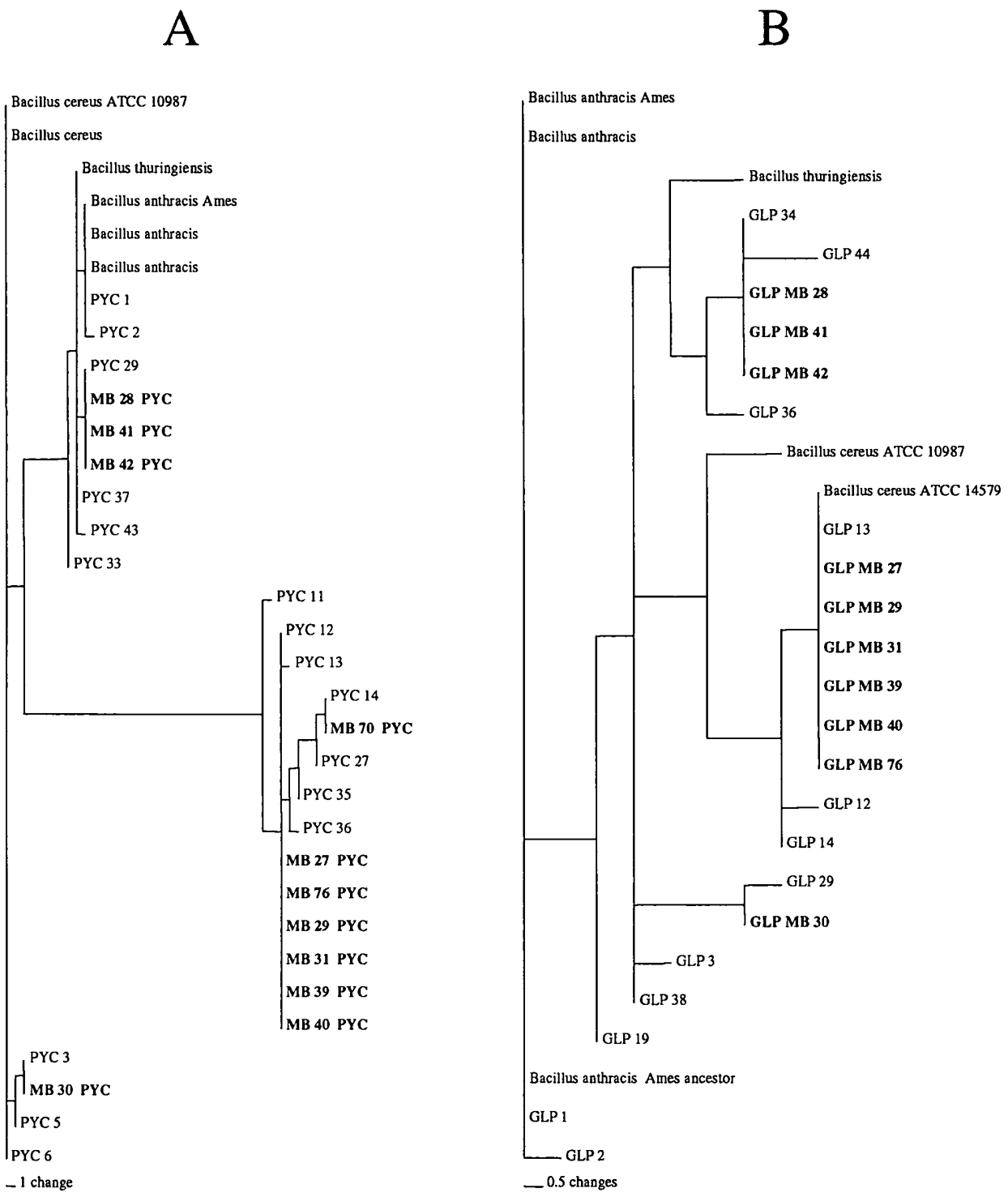

Figure 3

MLST sequence analysis trees. Maximum parsimony trees representing the B. cereus group, including types for *B. cereus*, *B. anthracis*, and *B. thuringiensis* and representatives from [29]. Relationships were established using two of the six primers generally used to distinguish the three *Bacillus* type bacteria within this group (GlyP, and PyC; [29]. Alignments generated from a 520 bp alignment of the GLP locus and a 520 bp alignment of the PYC locus. Isolates from honey bees shown in bold.

ing and multi-locus sequencing (GlyP, PyC) indicate that these isolates represent several distinct taxa from this group, although interference with *P. larvae* did not fall out with species identification. The high frequency of bees harboring bacteria from the *B. cereus* group suggests a stable symbiosis between bees and this taxon, perhaps helping to explain the fact that bees are more tolerant than many other insects toward *B. thuringiensis* [20]. Curiously, isolates from this group which shared both 16S haplotypes and sequence identity at the two protein-coding genes differ substantially in their ability to inhibit *P. larvae*. This variation could result from undetected genetic variation within subspecies, conditional activation of inhibitory substances, or a role for plasmids or other mobile elements in inhibition. Future experiments will help resolve the causes of conditional inhibition by *Bacillus cereus* subspecies.

There is a growing appreciation for the potentially beneficial roles of bacteria in honey bee colonies. Evans and Lopez [21] recently showed that non-pathogenic bacteria can stimulate the innate immune response of honey bee larvae, perhaps helping bees survive exposure to pathogens. Further, Reynaldi et al. [22] recently showed that bacteria isolated from bees in Argentina are inhibitory of the important bee fungal pathogen, *Ascosphaera apis*. It will be interesting to determine whether these species, in addition, are also inhibitory toward *P. larvae*, and to contrast the microbes associated with bees across different continents.

Bacterial symbionts likely play roles in individual and colony fitness across the social insects. Sharing of symbiotic bacteria is notoriously important for termite nutrition, and it is increasingly clear that both obligate and facultative symbioses are widespread in social insects. Recent evidence for a socially communicable defense against pathogens in termites [23] might indeed reflect sharing of bacteria among termite colony members, rather than the proposed induction of host-specific physiological changes.

Perhaps, as is apparent in the termites and ants [24,25], honey bees have evolved behavioral or physiological mechanisms to enhance the transmission of beneficial microbes, while battling those species which are pathogenic. This would indicate a delicate balancing act for bees and other social insects, allowing for the encouragement of beneficial species while maintaining barriers against exploitation by pathogens. If so, discrimination at the levels of behavior and individual immune responses might be used to bias the microbial biome within insect colonies toward mutualists and against parasites and pathogens. Beneficial symbionts can potentially be fed to developing bees as a prophylactic against disease [21], and can regardless be used to better understand the complexity of interactions between the microbial biota of bees. It will, in this vein, also be important to look more closely at transmission mechanisms of microbes within and between bee colonies.

Methods

Organisms

Bacteria were cultivated from a total of 341 honey bee larvae collected from four apiaries near the USDA-ARS Bee Research Laboratory, Beltsville, MD, USA from June-August, 2003 (Fig. 1). These larvae were collected from both mature honey bee colonies (n = 217 larvae in 51 colonies established at least one year prior to collection) and from colonies that had been newly established (134 larvae from 34 colonies). First-instar larvae were collected and reared for 24 h or 7d using an aseptic artificial diet, controlled temperature (34°C) and high humidity as described [19]. Larvae were frozen at -80°C prior to the cultivation and isolation of bacteria.

Inhibition assays

Individual larval bees were ground in 40 ul sterile H_2O at room temperature, using a disposable pestle. A filter-paper disk was impregnated with 20 ul from the resulting suspension. Each disk was centered on a standard Petri plate (100 × 15 mm) consisting of Brain-Heart Infusion (Difco) agar media containing 0.1 mg/ml thiamine hydrochloride as described [26]. Prior to placement of these disks, plates had been inoculated with a lawn of approximately 1×10^8 viable spores of *P. larvae*. These spores were isolated from diseased honey bee larvae collected in 2002 from a single bee colony in Berkeley, CA, U.S.A (BRL sample 230010; [25]). After 24 h incubation at 34°C, plates were scored for both bacterial growth and inhibition toward *P. larvae*. Bacterial growth was described as any bacteria on or contiguous to the paper disks that was atypical for *P. larvae*. Inhibition was defined as the radial distance between these paper disks and the first line of *P. larvae* growth. *P. larvae* inhibition was only observed in conjunction with growth of larval-derived bacteria (e.g., there were no signs of inhibition resulting from the larvae themselves by this assay). Inhibition was confirmed in all cases by replating collected bacterial cultures against a fresh lawn of *P. larvae*.

DNA extraction and sequencing

All bacterial colonies on or alongside paper disks were collected with a sterile wand. Approximately 10 mg vegetative cells of each sample was suspended in 300 ul of 30% glycerol solution then kept at -20°C. To isolate DNA, 50 ul from this suspension was mixed with 50 ul 10% Chelex-100 (Bio-Rad, Hercules, CA), then incubated at 72°C for 10–20 minutes before being placed on ice.

16S rRNA genes were amplified by PCR using universal eubacterial primers eu27.F and eu1495.R [27]; sequences 5'- gagagtttgatcctggctcag-3' and 5'- ctacggctaccttgttacga-3', respectively). Reaction mixes included 2 ul bacterial extract, 2 U Taq DNA polymerase with recommended buffer (Boehringer, Indianapolis, IN), 1 mM DNTP mix, 2 mM $MgCl_2$, and 0.2 μM of each primer. PCR was carried out on an MJ-Research PTC-100 thermal cycler using 30 cycles of 93°C 1 min, 54°C 1 min, 72°C 1 min. Bands of an appropriate size were confirmed by agarose gel electrophoresis, then PCR products were purified directly (Gene Pure). Products were sequenced using Big Dye 2.0 (Applied Biosystems, Carlsbad, CA) end-terminal cycle sequencing, followed by separation and analysis on an Applied Biosystems 3130 DNA Analysis machine. Sequencing was carried out in one direction from the 5' (eu27.F) end.

Sequence analyses

Sequences were checked and trimmed using the software program Sequencher (Gene Codes), then were aligned using the CLUSTALW algorithm, invoked by Omiga 2.0 (Oxford Molecular). Alignments also included a diverse array of bacterial species, including all of those previously identified from bees and representative members of the major bacterial clades. Alignments were exported to PAUP 4.0b10 (Sinauer) to generate phylogenetic hypotheses using a heuristic maximum-parsimony algorithm. Trees were generated for the entire data set, and for a data set limited to species found in the genus *Bacillus*. Sequences were also compared directly to all 16S rRNA sequences deposited in GenBank [28] using BLASTN, in August, 2005.

Multilocus sequences for Bacillus isolates

A large fraction of sequenced isolates were placed into the *Bacillus cereus* group by 16S rRNA similarity. To better resolve members of this group, isolates were sequenced at two protein-coding loci that offer informative sequence variation within this group [29]. Glycerol uptake factor protein (PCR and sequencing primers Glyp.F GCG TTT GTG CTG GTG TAA GT and GlyP.R CTG CAA TCG GAA GGA AGA AG) and Pyruvate carboxylase (primers PyC.F GCG TTA GGT GGA AAC GAA AG and PyC.R CGC GTC CAA GTT TAT GGA AT) genes were amplified by a three-step PCR regime of (94 30 s, 58 30 s, 72 1 m) × 30 and sequenced via Big-Dye N terminal sequencing as described above. Sequences were aligned with each other and voucher sequences from the Multilocus sequencing database http://pubmlst.org/bcereus/ using CLUSTAL, then were analysed by maximum parsimony using PAUP 4.0b.

Authors' contributions

JDE conceived of the project and both authors were involved with data collection, analysis, and writing.

Acknowledgements

We thank L. Decanini, I.B. Smith, J. Pettis, D. Lopez and A. Ulsamer for their help during this project. Funding was received from USDA-NRI grant 2002-02546 (JDE). Use of commercial names need not reflect endorsement of these products over others not mentioned.

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Microbiology (2004), 150, 2381–2390 DOI 10.1099/mic.0.27125-0

Biochemical characterization of different genotypes of *Paenibacillus larvae* subsp. *larvae*, a honey bee bacterial pathogen

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Paenibacillus larvae subsp. *larvae* (*P. l. larvae*) is the aetiological agent of American foulbrood (AFB), the most virulent bacterial disease of honey bee brood worldwide. In many countries AFB is a notifiable disease since it is highly contagious, in most cases incurable and able to kill affected colonies. Genotyping of field isolates of *P. l. larvae* revealed at least four genotypes (*AB*, *Ab*, *ab* and *αB*) present in Germany which are genotypically different from the reference strain DSM 7030. Therefore, based on these data, five different genotypes of *P. l. larvae* are now identified with genotype *AB* standing out with a characteristic brown-orange and circled two-coloured colony morphology. Analysing the metabolic profiles of three German genotypes (*AB*, *Ab* and *ab*) as well as of the reference strain using the Biolog system, a characteristic biochemical fingerprint could be obtained for each strain. Cluster analysis showed that while genotypes *Ab*, *ab* and the reference strain DSM 7030 are rather similar, genotype *AB* is clearly different from the others. Analysis of all isolates for plasmid DNA revealed two different plasmids present only in isolates belonging to genotype *AB*. Therefore, genotype *AB* is remarkable in all aspects analysed so far. Future analysis will show whether or not these differences will expand to differences in virulence.

Received 1 March 2004
Revised 30 March 2004
Accepted 7 April 2004

INTRODUCTION

The spore-forming, Gram-positive bacterium *Paenibacillus larvae* subsp. *larvae* (*P. l. larvae*) is the causative agent of American foulbrood (AFB), the most serious and fatal bacterial disease of honey bee larvae. AFB is highly contagious and has spread around the world. In many countries, AFB is a notifiable disease and, hence, control measures are often regulated by disease control orders. Since destruction of infected colonies is considered the only workable control measure by most authorities, AFB is causing considerable economic loss to beekeepers worldwide.

The oval-shaped spores represent the infectious stage of *P. l. larvae*. AFB is transmitted by spore-containing honey being fed to newly hatched larvae. So far, the only known host for *P. l. larvae* is honey bee larvae. The spores germinate in the midgut lumen. The vegetative forms of *P. l. larvae* then penetrate the gut epithelium and proliferate within the larval tissue. Within 72 h the bee larvae are reduced to tissue detritus, which forms a glue-like colloid (rope stage). Later still the larval remains dry down to a scale adhering to the side of the cell. This scale is highly infectious since it contains billions of spores (Bailey & Ball, 1991; Gregorc & Bowen, 1998).

Abbreviations: AFB, American Foulbrood; rep-PCR, repetitive element PCR; *P. l. larvae*, *Paenibacillus larvae* subsp. *larvae*; *P. l. pulvifaciens*, *Paenibacillus larvae* subsp. *pulvifaciens*.

Recent genotyping of German field isolates of *P. l. larvae* revealed at least four different genotypes, named *AB*, *Ab*, *ab* and *αB* (Genersch & Otten, 2003). Here we present data on the further characterization of the genotypes *AB*, *Ab* and *ab*, and of the reference strain DSM 7030/ATCC 9545. Biochemical fingerprinting was performed using the Biolog system. This system involves the determination of the metabolism of 95 carbon sources in a microtitre plate format. Since the metabolism of bacteria is adapted to their natural environment or host, each bacterium prefers or uses particular carbon sources. Hence, determination of the metabolic profile of a micro-organism can be used for identifying and characterizing the organism.

Earlier reports show that some isolates of *P. l. larvae* harbour plasmid DNA (Benada *et al.*, 1988; Bodorova-Urgosikova *et al.*, 1992). Therefore, we analysed all isolates for the presence of extrachromosomal DNA to see whether or not the occurrence of plasmid DNA might be an additional feature suitable for typing purposes and epidemiology.

METHODS

Bacterial isolates. The *P. l. larvae* reference strain DSM 7030 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany. *P. l. larvae* DSM 7030 is identical to reference strain ATCC 9545.

Paenibacillus larvae subsp. *pulvifaciens* (*P. l. pulvifaciens*) reference strains DSM 8442 and DSM 8443 were obtained from DSMZ. *P. l. pulvifaciens* DSM 8442 and DSM 8443 are synonyms for *P. l. pulvifaciens* reference strains NRRL NRS 1683 and NRRL NRS 1684, respectively.

Eighty-six German field isolates from *P. l. larvae* were isolated from honey samples originating from 86 AFB-positive hives diagnosed in the course of foulbrood monitoring programs between 2000 and 2003 (Table 1). Diagnosis was based on clinical symptoms as well as on isolation, cultivation and absolute identification of the causative agent, *P. l. larvae*. Honey samples had been stored at 4 °C until *P. l. larvae* was cultivated for scientific purposes from these samples on bacterial plates. Two representatives of the recently identified *P. l. larvae* genotype *AB* (Genersch & Otten, 2003) have been deposited at the DSMZ (accession numbers DSM 16115, DSM 16116).

Bacterial culture. *P. l. pulvifaciens* reference strains DSM 8442 and DSM 8443 as well as *P. l. larvae* reference strain DSM 7030 were cultured on Columbia sheep blood agar plates exactly as directed by DSMZ.

The culture of *P. l. larvae* from honey samples was performed essentially as previously described (Genersch & Otten, 2003). Briefly, for growth of spore-forming bacteria, honey samples were solubilized overnight at 37 °C. Subsequently, samples were diluted in double-distilled water to obtain a 50 % (w/v) honey solution. To select for spores, samples were incubated at 90 °C for 6 min. Samples were allowed to cool down at room temperature prior to plating them (200 µl per plate) onto Columbia sheep blood agar plates. Three plates were prepared from each sample. Plates were incubated at 37 °C and evaluated for bacterial growth after 3 and 6 days. After 6 days, *P. l. larvae*-like colonies were identified by catalase and Plagemann tests as well as by PCR detection prior to subsequent detailed analysis.

For further analysis, all isolates were stored as bacterial suspensions in 25 % glycerol in BHI (brain heart infusion) broth at −70 °C.

Biochemical analysis of *P. l. larvae* reference strain DSM 7030 was performed by subculturing this strain and taking 24 independent subcultures.

Catalase and Plagemann tests. For absolute identification, colonies with a *P. l. larvae*-like morphology were further analysed by catalase and Plagemann tests. For the catalase test part of the colony in question was transferred to a microscopic slide using a wooden stick and mixed with a drop of 3 % H_2O_2. Production of air bubbles is indicative for catalase activity, whereas no air bubbles indicates a lack of catalase activity. For the Plagemann test (Plagemann, 1985), the liquid part of Columbia sheep blood agar slants was inoculated with part of the bacterial colony in question. The tube was air-tight sealed with Parafilm and incubated at 37 °C for 10 days. Subsequently, the liquid part was analysed for the presence of spores and giant whips by phase-contrast microscopy. *P. l. larvae* is characterized by the lack of catalase activity and giant whips occurring during sporulation (Ritter, 1996; Hansen & Brodsgaard, 1999).

***P. l. larvae*-specific PCR.** For PCR identification of bacterial colonies grown on agar plates, part of the colony in question was resuspended in 50 µl double-distilled water and subsequently incubated at 90 °C for 15 min. Probes were centrifuged at 5000 *g* for 10 min. The supernatant containing the DNA was transferred to a new tube and directly used for PCR analysis. PCR analysis was based on 16S rDNA sequences of *P. l. larvae* (accession numbers AY030079 for strain NRRL B-3555 and X60619 for strain ATCC 9545) and on the partial sequence of the gene for a 35 kDa metalloprotease from *P. l. larvae* (AF111421). Primer sequences were designed using MacVector 6.5 software and compared with published sequences in the GenBank databases using BLAST (Altschul *et al.*, 1990): Pll-16S E1, 5′-GCAAGTCGAGCGGACCTTGTG-3′; Pll-16S E2, 5′-AAACCGGTCAGAGGGATGTCAAG-3′; Pll-16S F6, 5′-GCACTGGAAACTGGGAGACTTG-3′; Pll-16S B11, 5′-CGGCTT-TTGAGGATTGGCTC-3′; Pll-MP F3, 5′-CGGGCAGCAAATCGTAT-TCAG-3′; Pll-MP B1, 5′-CCATAAAGTGTTGGGTCCTCTAAGG-3′.

PCR analyses were carried out in a final volume of 25 µl consisting of 1 × Qiagen reaction buffer, 250 µM dNTPs (dATP, dCTP, dGTP, dTTP), 10 µM primer and 0·3 U HotStarTaq polymerase (Qiagen). Concentrations of $MgCl_2$ were adjusted so that all three reactions could be performed at a final annealing temperature of 56 °C: 2·3 mM for primer pair Pll-16S E1/E2, 1·7 mM for primer pair Pll-16S F6/B11 and 1·5 mM for primer pair Pll-MP F3/B1. After the initial activation step (15 min, 95 °C), the reaction conditions for the touchdown PCR were as follows. All denaturation steps were performed at 94 °C for 30 s, all elongation steps were performed at 72 °C for 30 s, and all annealing steps were performed for 1 min. For annealing, temperatures of 66, 62 and 58 °C were used and 5 cycles were run at each temperature. At the final annealing temperature of 56 °C, 30 cycles were run followed by a final elongation step at 72 °C for 8 min. Five microlitres of the PCR samples was analysed on a 0·8 % agarose gel. The DNA bands were stained with ethidium bromide and visualized by UV light.

The expected lengths of specific amplicons generated with Pll-16S E1/E2, Pll-16S F6/B11 and Pll-MP F3/B1 were 965, 665 and 273 bp, respectively. For all *P. l. larvae* genotypes, specificity of amplicons was verified by sequencing (Medigenomix, Germany). When tested for specificity with *P. l. pulvifaciens* reference strains DSM 8442 and DSM 8443, none of the primers generated any PCR product (Fig. 1). Specific PCR products were not generated when control PCR analyses were performed with reference strains for *Paenibacillus alvei* (DSM 29), *Paenibacillus apiarius* (DSM 5581, DSM 5582, DSM 5612), *Paenibacillus polymyxa* (DSM 36), *Bacillus licheniformis* (DSM 13), *Bacillus mycoides* (DSM 299), *Bacillus thuringiensis* (DSM 6029) and other unidentified bacilli isolated from honey samples (data not shown).

Repetitive element PCR (rep-PCR) analysis. Preparation of bacterial DNA for fingerprinting and subsequent rep-PCR analysis of bacterial isolates was performed essentially as described previously (Genersch & Otten, 2003). In brief, *P. l. larvae* DNA suitable for rep-PCR DNA fingerprinting was isolated from colonies grown on culture plates using 6 % InstaGene matrix (Bio-Rad) following the instructions of the manufacturer. The DNA sequences of the primers used for DNA fingerprinting were as follows (Versalovic *et al.*, 1994): 5́-CTACGGCAAGGCGACGCTGACG-3 (BOX A1R), 5́-CCGCCGTTGCCGCCGTTGCCGCCG-3́ (MBO REP1). PCR analyses were carried out in a final volume of 25 µl consisting of 1 × reaction buffer (Qiagen) and a final concentration of 2·5 mM $MgCl_2$, 250 µM dNTPs (dATP, dCTP, dGTP, dTTP), 10 µM primer and 0·3 U HotStarTaq polymerase (Qiagen). The reaction conditions were as follows. After the initial activation step (15 min, 95 °C), 35 cycles at 94 °C for 1 min, 53 °C for 1 min and 72 °C for 2·5 min were run followed by a final elongation step at 72 °C for 10 min. Five microlitres of the PCR samples was analysed on a 0·8 % agarose gel. The DNA bands were stained with ethidium bromide and visualized by UV light.

Biochemical fingerprinting (Biolog system). The Biolog system (obtained through Oxoid) is a carbon source test, where the ability of a bacterial isolate to metabolize 95 different carbon sources is used for identification purposes. Cultivation and preparation of *P. l. larvae* isolates for metabolic analysis using the Biolog system were performed according to the manufacturer's instructions for spore-forming, Gram-positive rods, with minor modifications to meet the growth requirements of *P. l. larvae*. Briefly, single pure colonies of

Table 1. List of isolates used in this study

All field isolates from *P. l. larvae* were isolated from honey originating from AFB-positive hives diagnosed in the course of foulbrood monitoring programs in Germany between 2000 and 2003. Honey samples had been collected close to the brood nest from brood combs showing clinical symptoms of AFB. DSM 7030 is a *P. l. larvae* reference strain (identical to *P. l. larvae* strain ATCC 9545) obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany).

Isolate	*P. l. larvae* genotype				Year of isolation	Isolate	*P. l. larvae* genotype				Year of isolation
	AB	*Ab*	*ab*	*aβ*			AB	*Ab*	*ab*	*aβ*	
00-775.1	+				2000	02-122		+			2002
00-775.2	+				2000	02-180		+			2002
00-869	+				2000	03-019		+			2003
00-897	+				2000	03-159		+			2003
00-1163.1	+				2000	00-087			+		2000
00-1163.2	+				2000	01-145			+		2001
01-000.1	+				2001	01-247			+		2001
01-000.2	+				2001	01-248			+		2001
01-000.3	+				2001	01-249			+		2001
01-649.1	+				2001	01-281			+		2001
01-649.2	+				2001	01-282			+		2001
01-1714	+				2001	01-283			+		2001
02-009	+				2002	01-337			+		2001
02-334	+				2002	01-391			+		2001
03-016.1	+				2003	01-402			+		2001
03-016.2	+				2003	01-454			+		2001
03-098	+				2003	01-455			+		2001
03-195	+				2003	01-456			+		2001
03-199	+				2003	01-1707.1			+		2001
03-201	+				2003	01-1707.2			+		2001
03-476	+				2003	01-1709.1			+		2001
03-478	+				2003	01-1709.2			+		2001
03-479	+				2003	02-127			+		2002
03-518	+				2003	02-128			+		2002
03-522	+				2003	02-129			+		2002
03-525	+				2003	02-130			+		2002
01-358		+			2001	02-141			+		2002
01-440		+			2001	02-149			+		2002
02-060		+			2002	02-179			+		2002
02-065		+			2002	02-250			+		2002
02-066		+			2002	02-360			+		2002
02-067		+			2002	03-015			+		2003
02-070		+			2002	03-017			+		2003
02-075		+			2002	03-121			+		2003
02-076		+			2002	03-122			+		2003
02-081		+			2002	03-125a			+		2003
02-083		+			2002	03-125b			+		2003
02-108		+			2002	03-125c			+		2003
02-111.1		+			2002	03-125d			+		2003
02-111.2		+			2002	03-126			+		2003
02-113		+			2002	03-128			+		2003
02-117		+			2002	03-384			+		2003
02-120		+			2002	DSM 7030				+	Reference strain
02-121		+			2002						

P. l. larvae were subcultured on BUG-M-T agar plates (bacterial universal growth agar supplemented with 0·25 % maltose and swapped with thioglycolate) with one colony per plate. Using sterile wooden sticks a special streaking technique was performed resulting in a 'plus' sign on the centre of the plate. The goal of this technique is to restrain cell growth to two thin lines so that the cells along the edges

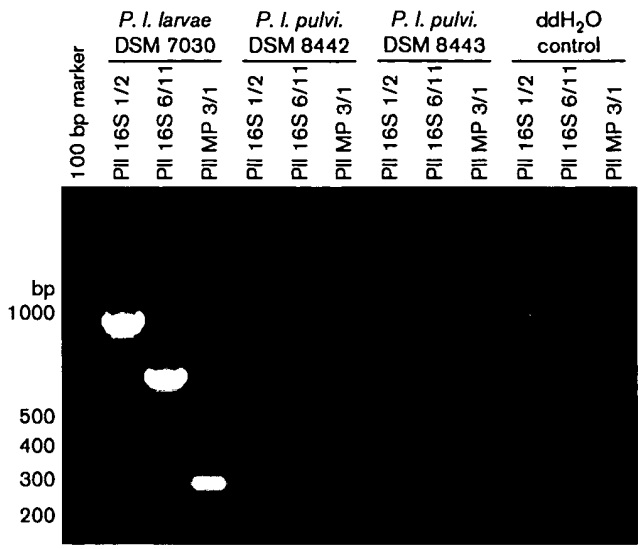

Fig. 1. PCR identification of *P. l. larvae*. PCR analysis of *P. l. larvae* reference strain DSM 7030 and *P. l. pulvifaciens* reference strains DSM 8442 and DSM 8443 using primer pairs PII-16S E1/E2 (PII 16S 1/2) and PII-16S F6/B11 (PII 16S 6/11) for amplification of 16S rDNA fragments and primer pair PII-MP F3/B1 (PII MP 3/1) for amplification of a metalloproteinase gene segment. PCR products were analysed on a 0·8% agarose gel in the presence of appropriate molecular size markers.

have a good supply of food. This keeps the cells in an active state and decreases sporulation. For the analysis of one isolate, eight pure colonies of this isolate were subcultured on eight BUG-M-T plates. Growth of bacteria at 37 °C was continued for 48 h since *P. l. larvae* is a slow-growing bacterium. Subsequently, the inoculum was prepared by taking only those colonies starting at the ends of the 'plus' sign to half way down the junction of the two lines constituting the 'plus' sign. Bacteria from the centre or close to the centre must not be taken. Colonies were picked up with a wooden stick and rubbed around the walls of an empty, sterile dry glass tube. The bacterial film was suspended in 5 ml inoculation solution (GN/GP IF; Oxoid) to obtain a homogeneous mixture. After adding the remaining fluid (10 ml), turbidity was adjusted to match the turbidity standard (Oxoid) at 28±2% turbidity (OD=0·55). Subsequently, a GP2 MicroPlate (Oxoid) was inoculated with 150 µl bacterial suspension per well and incubated at 37 °C for 24 h. Metabolic activity was determined by reading the plates (end point reading method) in a microplate reader (EL800; BIO-TEK Instruments) with a primary wavelength of 590 nm and a reference wavelength of 750 nm. Positive wells were then entered manually into the Biolog MicroLogI (release 4.20) software after choosing plate type (GP2), strain type (GP-ROD SB) and incubation time (16–24 h). Interpretation of the results and identification of the bacterial strain in question was performed automatically by the software. The Biolog database does include a standard profile for *P. l. larvae* (Table 2) and, therefore, is able to identify *P. l. larvae*. The software does not include *P. l. pulvifaciens*.

Cluster analysis was performed by Biolog MicroLog3 software after manually entering the data. The dendrogram was generated using a modified UPGMA analysis (Biolog) where the algorithm uses DIST values to generate the branching structure of the dendrogram.

Preparation of plasmid DNA. Frozen bacterial suspensions were thawed, plated on Columbia sheep blood agar plates and allowed to grow for 3 days at 37 °C. Colonies were scraped off and resuspended in 300 µl BHI broth. Up to six plates were pooled to yield a sufficient amount of bacteria for plasmid preparation. Subsequently, bacteria were pelleted by centrifugation at 5000 *g* for 10 min. The bacterial pellet was used for plasmid preparation performed with the QIAprep Spin Miniprep kit (Qiagen) by exactly following the manufacturer's protocol. A 12 µl sample of each eluent was analysed on a 0·8% agarose gel. The DNA bands were stained with ethidium bromide and visualized by UV light.

Restriction analysis of plasmid DNA. Depending on band intensity, 2–8 µl of each eluent containing plasmid DNA was used for restriction analysis. Restriction reactions were carried out in a final volume of 10 µl using 10 U of the corresponding restriction enzyme together with the appropriate reaction buffer. Reactions were incubated at 37 °C for 30 min. Subsequently, 2 µl 6× DNA loading buffer was added and the reactions were analysed on a 0·8% agarose gel. The DNA bands were stained with ethidium bromide and visualized by UV light.

RESULTS

rep-PCR analysis of *P. l. larvae*

By analysing German *P. l. larvae* isolates using rep-PCR fingerprinting performed with primers BOX A1R and MBO REP1, three BOX A1R and two MBO REP1 patterns (*A*, *a*, *α* and *B*, *b*, respectively) were characterized recently (Genersch & Otten, 2003). Differential combinations of these patterns were found, resulting in the identification of the four genetic subgroups *AB*, *Ab*, *ab* and *αB* (Genersch & Otten, 2003). rep-PCR analysis of *P. l. larvae* reference strain DSM 7030 (identical to ATCC 9545) now revealed a third MBO REP1 pattern (*β*), with the characteristic band around 1000 bp missing, and its affiliation to BOX A1R-group *a* (Fig. 2). Hence, *P. l. larvae* DSM 7030 belongs to a new genotype *aβ*, hitherto not found as a field isolate in Germany.

Colony morphology of *P. l. larvae*

Colonies of *P. l. larvae* subgroups *Ab*, *ab* and *aβ* have a whitish to greyish, somewhat transparent and slightly glistening appearance (Fig. 3a). In contrast, isolates belonging to subgroup *AB* show a considerable deviation from this normal colony morphology. Small colonies of *AB* are unicoloured red-brown or white without being transparent, whereas bigger colonies mostly show concentric brownish and whitish circles with the outermost circle always being orange-brown (Fig. 3b). Both the unicoloured and the circled colony phenotypes were unambiguously identified as *P. l. larvae* by Plagemann and catalase tests as well as by *P. l. larvae*-specific PCR detection. Both phenotypes have identical rep-PCR patterns characteristic for subgroup *AB* (data not shown). The phenotypic differences are not stable in the sense that serial cultivation of single white, single red-brown and single brownish-circled colonies would result in only white, red-brown and brownish-circled colonies, respectively. These variants rather split up again into the

Table 2. Metabolic profiles of different strains of *P. l. larvae* as determined by using the Biolog system

Results are expressed as a percentage of positive reactions obtained for each carbon source. Only those carbon sources used by *P. l. larvae* are given.

Well:	A5	A10	A11	B5	B11	C6	C11	D1	D4	E2	E3	F6	F9	G3	G12	H1	H2	H3	H4	H5	H6	H7	H8
Carbon source:	Glycogen	*N*-Acetyl-D-glucosamine	*N*-Acetyl-β-D-mannosamine	D-Fructose	α-D-Glucose	D-Mannose	3-Methyl-D-glucose	β-Methyl-D-glucoside	D-Psicose	D-Trehalose	Turanose	Pyruvic acid methyl ester	Pyruvic acid	L-Alanine	Glycerol	Adenosine	2'-Deoxy-adenosine	Inosine	Thymidine	Uridine	Adenosine-5'-monophosphate	Thymidine-5'-monophosphate	Uridine-5'-monophosphate
Biolog standard	5	100	15	0	50	20	20	20	0	100	15	70	90	10	100	20	5	10	20	30	10	15	20
Strain DSM 7030 ($n=24$)*	0	100	13	0	54	0	0	38	0	100	0	75	96	0	100	58	21	17	100	58	50	83	79
Genotype *AB* ($n=26$)†	0	100	19	100	58	38	0	0	96	92	0	96	100	0	0	69	73	0	96	96	0	0	0
Genotype *Ab* ($n=22$)†	0	100	0	0	86	0	0	27	0	100	0	77	100	0	100	86	91	59	100	100	100	100	100
Genotype *ab* ($n=38$)†	0	100	0	0	50	0	0	24	0	100	0	97	100	0	87	97	97	84	100	87	95	100	97

P. l. larvae reference strain DSM 7030 was obtained from the DSMZ culture collection. Twenty-four independent subcultures of this strain were used for Biolog characterization to obtain a value for 'percentage of positive reactions'.

†*P. l. larvae* genotypes *AB*, *Ab* and *ab* were isolated from different outbreaks of AFB in Germany between 2000 and 2003. Different and independent field isolates (*n* given in brackets) were used for Biolog characterization to obtain the percentage of positive reactions.

same three variants (data not shown). Therefore, not only the deviation, but also the variability of colony pigmentation has to be considered as a stable and characteristic morphological feature of colonies of subgroup *AB*.

Biochemical characterization of *P. l. larvae*

The Biolog system takes into account that the metabolic profile of a given bacterium is not a static feature but rather differs between different isolates or independent cultures of one isolate. Hence, the standard profiles are given as percentages of positive reactions for each carbon source. Using the Biolog system (Oxoid) we analysed the metabolic pattern of *P. l. larvae* subgroups *AB*, *Ab*, *ab* and *aβ* (Table 2). Genotypes *AB*, *Ab* and *ab* were represented by 26, 22 and 38 different and independent field isolates, respectively. To also obtain ratios of positive reactions for genotype *aβ*, *P. l. larvae* reference strain DSM 7030 was tested by taking 24 independent subcultures of this strain. All 110 samples were unambiguously identified as *P. l. larvae* by the Biolog identification software. The standard metabolic pattern established for *P. l. larvae* by Biolog and used for identification is also given in Table 2 (Biolog standard).

All cultures of strain DSM 7030 (genotype *aβ*) were able to metabolize *N*-acetyl-D-glucosamine, D-trehalose, glycerol and thymidine, whereas D-fructose, D-mannose, 3-methyl-D-glucose, D-psicose, turanose and L-alanine were not accepted as carbon source. The other carbon sources given in the table were differentially used by DSM 7030. All isolates of genotype *AB* were able to metabolize *N*-acetyl-D-glucosamine, D-fructose and pyruvic acid, but none was able to degrade 3-methyl-D-glucose, β-methyl-D-glucoside, turanose, L-alanine, glycerol, inosine, adenosine-5'-monophosphate, thymidine-5'-monophosphate and uridine-5'-monophosphate. The other carbon sources given in the table were used by 19–96 % of all *AB* isolates. All isolates of genotypes *Ab* and *ab* were able to digest *N*-acetyl-D-glucosamine, D-trehalose, pyruvic acid, thymidine and thymidine-5'-monophosphate. In addition, 100 % of genotype *Ab* metabolized glycerol, uridine, adenosine-5'-monophosphate and uridine-5'-monophosphate. *N*-Acetyl-β-D-mannosamine, D-fructose, D-mannose, 3-methyl-D-glucose, D-psicose, turanose and L-alanine were never used by any isolate of genotypes *Ab* and *ab*. All other carbon sources given in the table were differentially metabolized by isolates belonging to these subgroups. The

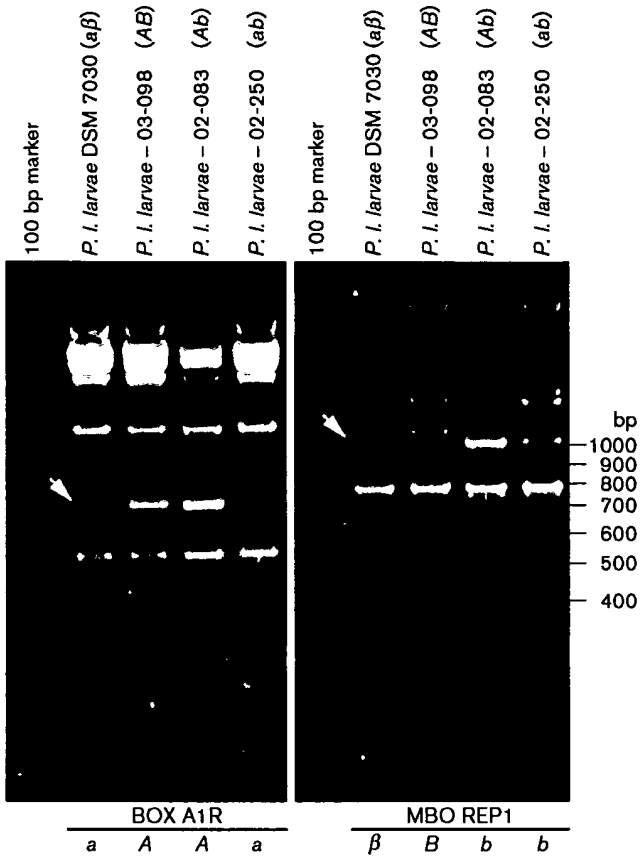

Fig. 2. *P. l. larvae* reference strain DSM 7030 belongs to the newly identified genotype *aβ*. Using BOX A1R and MBO REP1 primers, *P. l. larvae* reference strain DSM 7030 was analysed by rep-PCR. For comparison, rep-PCR patterns from recently identified *P. l. larvae* genotypes *AB*, *Ab* and *ab* are also shown. Differences in banding pattern between the BOX groups *A* and *a* and between the MBO groups *β*, *B* and *b* are highlighted by arrows. By combining the different BOX and MBO patterns for a single isolate the four genetic subgroups *aβ*, *AB*, *Ab* and *ab* are revealed.

relationships between the four different analysed strains in comparison to the Biolog standard profile and other metabolically related bacteria are given in the dendrogram in Fig. 4. Genotypes *Ab*, *aβ* and *ab* are rather similar to each other, but nevertheless distinct from the Biolog standard metabolic profile. The metabolic distance between genotype *AB* and the other genotypes becomes abundantly clear.

Screening for and characterization of plasmid DNA harboured by *P. l. larvae*

Inspired by earlier reports on some *P. l. larvae* isolates harbouring plasmids (Benada *et al.*, 1988; Bodorova-Urgosikova *et al.*, 1992; Drobnikova *et al.*, 1994), we screened all our isolates for extrachromosomal DNA. We found two different plasmid molecules of 9·4 and 11·0 kb, denoted pPll9.4 and pPll11.0, respectively (Fig. 5). The

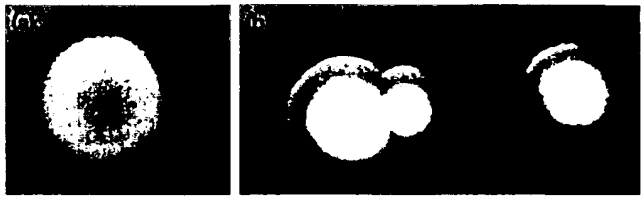

Fig. 3. Characteristic colony morphology of *P. l. larvae*. (a) The normal morphology of colonies of *P. l. larvae* is whitish to greyish, somewhat transparent and slightly glistening. (b) In contrast, colonies of genotype *AB* show orange-brown circles and clearly differ from normal colony morphology. Colonies shown are 6 days old.

presence of plasmids was restricted to isolates belonging to genotype *AB*; no plasmid DNA could be found in the reference strain DSM 7030 or in *Ab* and *ab* strains (Fig. 5). Normally, one isolate harboured either pPll9.4 or pPll11.0, but in one case both plasmids were found together (Fig. 5, lane 7). The characteristic restriction cleavage pattern of the predominant pPll9.4 obtained with restriction enzymes *Hin*dIII, *Eco*RI and *Xba*I is shown in Fig. 6(a). As estimated from the electrophoretic mobilities, *Hin*dIII generates six fragments of 500, 600, 1150, 2300, 2400 and 2450 bp, the latter two migrating as a double band. In contrast, *Eco*RI generates only two fragments of 850 and 8550 bp. Plasmid pPll9.4 is linearized by *Xba*I resulting in a band migrating at 9400 bp, correlating with the size of the plasmid as calculated from the sum of the fragments generated with *Hin*dIII and *Eco*RI. Plasmid pPll11.0

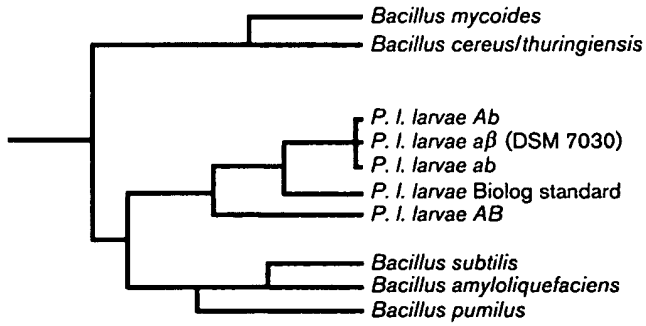

Fig. 4. Dendrogram showing the metabolic relationship between *P. l. larvae* genotypes *Ab*, *aβ*, *ab* and *AB* in relation to the Biolog standard and other metabolically related bacilli. Multiple samples of the different genotypes were analysed for their metabolic profile using the Biolog system. Based on the results obtained, a characteristic metabolic pattern for each genotype could be defined. These characteristic profiles were used to construct a dendrogram via a modified UPGMA analysis (Biolog). Genotypes *Ab*, *aβ* and *ab* are distinct, but nevertheless quite similar. In contrast, genotype *AB* is clearly different from all other genotypes as well as from the Biolog standard.

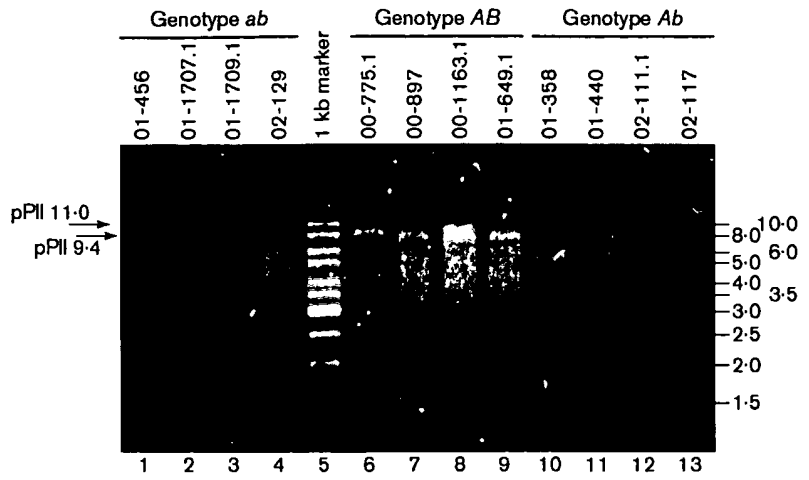

Fig. 5. Plasmids isolated from *P. l. larvae*. All isolates analysed in detail in this study were also screened for the presence of extrachromosomal DNA. Isolates belonging to genotype *AB* harboured two different plasmids of 9·4 and 11·0 kb, named pPll9.4 and pPll11.0, respectively. No plasmid DNA could be isolated from the other genotypes. Representative results are shown.

obviously results from a 1600 bp insertion into the 2400 bp *Hin*dIII fragment which is part of the 8550 bp *Eco*RI fragment as can be deduced from comparing the restriction cleavage patterns of pPll9.4 and pPll11.0 (Fig. 6b). This insertion contains an additional *Xba*I site since cleavage of pPll11.0 with *Xba*I does not linearize the plasmid but generates two fragments of 6000 and 5000 bp.

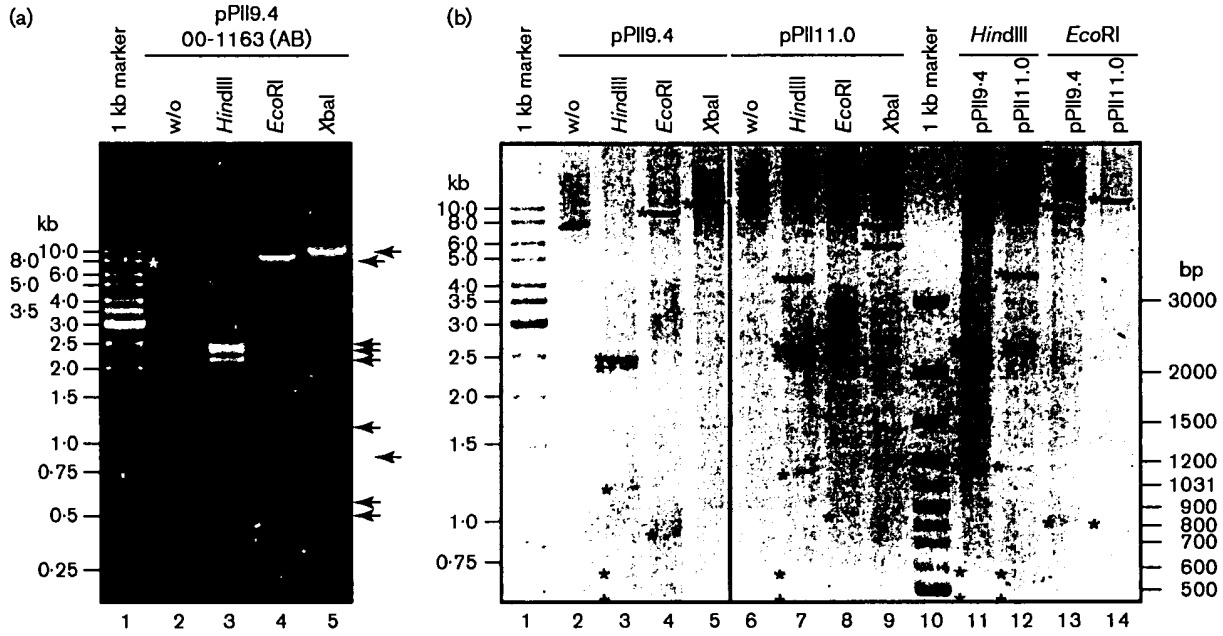

Fig. 6. Restriction analysis of plasmids pPll9.4 and pPll11.0. (a) Restriction analysis of plasmid pPll9.4 was performed with restriction enzymes *Hin*dIII, *Eco*RI and *Xba*I. Cleavage with *Hin*dIII yielded six fragments of 500, 600, 1150, 2300, 2400 and 2450 bp (lane 3). Obviously, pPll9.4 has only two *Eco*RI sites giving rise to two fragments of 850 and 8550 bp (lane 4). *Xba*I linearized pPll9.4; accordingly, the *Xba*I fragment of pPll9.4 migrates around 9400 bp (lane 5). (b) Analysing both plasmids pPll9.4 and pPll11.0 by restriction enzyme cleavage revealed that for all three enzymes the restriction patterns of pPll9.4 (lanes 2–5) and pPll11.0 (lanes 6–9) show differences resulting from a 1600 bp insertion into the 2400 bp *Hin*dIII fragment (lanes 11, 12) and into the 8550 bp *Eco*RI fragment (lanes 13, 14) respectively. In addition, the insertion contains a second *Xba*I site, leading to two *Xba*I fragments of 6000 and 5000 bp (lane 9) as opposed to the single *Xba*I fragment of pPll9.4 (lane 5). The *Hin*dIII double band of pPll9.4, migrating around 2450 bp, as well as fragments smaller than 900 bp, are hardly visible in (b) but clearly visible in (a). w/o, No enzyme.

DISCUSSION

AFB is the most serious and fatal bacterial disease of honey bee larvae. It is caused by the spore-forming bacterium *P. l. larvae*. Absolute identification of *P. l. larvae* requires the bacterium be grown in the laboratory (Office International des Epizooties, 2000). Diagnosis of AFB then relies on visually identifying *P. l. larvae* colonies grown on agar plates. Only suspect colonies are further analysed to confirm the initial identification. Normal colony morphology of *P. l. larvae* is described as whitish to greyish, somewhat transparent and slightly glistening (Bailey & Ball, 1991). In this study, we demonstrated that the above description does not hold true for all isolates of *P. l. larvae*. Genotype *AB*, identified among German field isolates (Genersch & Otten, 2003), is characterized by a striking circled whitish and brownish or unicoloured red-brown or white morphology. Although a pigmented variant of *P. l. larvae* has been described (Drobnikova *et al.*, 1994), in more recent studies production of an orange pigment has only been attributed to *P. l. pulvifaciens* (Heyndrickx *et al.*, 1996). Here, we again provide evidence for the existence of a pigmented phenotype of *P. l. larvae*. While Drobnikova *et al.* (1994) failed to find any feature discriminating their pigmented variant from the non-pigmented strains, we can show that the ability to produce pigmented colonies is characteristic for *P. l. larvae* genotype *AB*. The importance of this observation lies within the fact that not knowing this particular phenotype might lead to false-negative diagnostic results. False-negative diagnostic results are a threat to beekeeping since AFB is highly contagious and is able to kill affected colonies. Control measures for AFB depend on correct and early diagnosis of this disease. Therefore, knowledge of all possible phenotypic variations of *P. l. larvae* colonies is vital for exact diagnosis.

Earlier reports on biochemical characterization of *P. l. larvae* using traditional macro (Jelinski, 1985; Alippi & Aguilar, 1998) or commercial micro methods (Carpana *et al.*, 1995; Dobbelaere *et al.*, 2001) showed the usefulness of such tests for the classification of *P. l. larvae*, although the results obtained were somewhat contradictory. It was suggested that these differences are due to the different systems used (Dobbelaere *et al.*, 2001). Here we present evidence that the discrepancies between different studies on the biochemical properties of *P. l. larvae* are rather due to genotype-specific differences. We analysed the metabolic pattern of four different genotypes of *P. l. larvae* (*AB*, *Ab*, *ab* and *aβ*) by using the Biolog system. The system involves the determination of the metabolism of 95 different carbon sources. Interpretation of positive reactions is performed via an ELISA reader. Allotting the metabolic fingerprints to the different genotypes revealed characteristic patterns for each genotype (Table 2). To our knowledge this is the first time that genotype-specific metabolic profiles could be defined for *P. l. larvae*, indicating that differences in genotype correlate with differences in biochemical phenotype. When compared to other genotypes, *P. l. larvae* genotype *AB* exhibits the most striking metabolic pattern, since it is the only strain able to metabolize the carbohydrates D-fructose (100 %) and D-psicose (96 %), and the only strain unable to use glycerol as carbon source. The other strains, of genotypes *Ab*, *ab* and *aβ*, also show characteristic metabolic patterns, but are nevertheless more similar to each other. Analysing the metabolic pattern of two reference strains for *P. l. pulvifaciens*, DSM 8442 and DSM 8443, resulted in no identification using the Biolog software, since their biochemical fingerprint differed in more than 20 carbon sources from the profiles of *P. l. larvae* (J. Kilwinski, M. Peters, A. Ashiralieva & E. Genersch, unpublished results). Therefore, the Biolog system allows not only the identification of *P. l. larvae*, but also the definite discrimination between genotype *AB* and the other genotypes. Since the Biolog system is used in microbiological diagnosis, this result will be of diagnostic relevance if differences in virulence can be assigned to differences in genotype.

Our results show that the characteristic metabolic patterns always contain some variables for each genotype. Hence, groupings based on biochemical properties where these properties are understood as static traits will lead to results which hardly correlate with genotyping.

Comparing our results with the Biolog standard we can conclude that at least genotype *AB* was not included when establishing the standard metabolic fingerprint for *P. l. larvae*. D-Fructose and D-psicose are both given 0 %, meaning that it was never accepted as a carbon source by any isolate included in the survey. In contrast, D-fructose and D-psicose are metabolized by 100 and 96 %, respectively, by isolates belonging to genotype *AB*. Furthermore, some strains included in the Biolog standard are not represented in our study, since we never found any isolate able to metabolize 3-methyl-D-glucose, turanose or L-alanine. The exact metabolic relationships between the different genotypes of *P. l. larvae* in relation to the Biolog standard are given in the dendrogram (Fig. 4). The distance in biochemical phenotype between genotype *AB* and the other genotypes and, in contrast, the relative closeness of genotypes *Ab*, *ab* and *aβ* become obvious.

Jelinski (1985) distinguished seven biochemical types (I–VII) according to seven possible combinations of three biochemical properties: reduction of nitrate to nitrite, hydrolysis of mannitol and acid production from salicin. The same study revealed the ability to metabolize glycerol as a consistent feature of *P. l. larvae*. Based on our analysis using the Biolog system, this holds true for genotypes *Ab*, *ab* and the reference strain DSM 7030. In contrast, no isolate belonging to genotype *AB* was able to use glycerol as carbon source. It has been reported that comparison between the biochemical type (I–VII; Jelinski, 1985) and the genotype of isolates rendered no obvious link between both features (Alippi & Aguilar, 1998). This result is in disagreement with our results showing a clear link between biochemical and rep-PCR fingerprints. In the study performed by Alippi & Aguilar (1998) genotyping is based on rep-PCR performed with primers BOX A1R and REP (REP1R.I and REP2-I), a

primer combination having less discriminatory power than BOX A1R combined with MBO REP1 (Genersch & Otten, 2003), as used in our study. Therefore, it is likely, that the genotypes defined by Alippi & Aguilar (1998) would split up if MBO REP1 primers were used instead of REP primers, possibly allowing a better correlation between biochemical type and genotype. Furthermore, the discriminatory power of only three metabolic properties is quite poor as compared to the Biolog system where the metabolism of a total of 95 carbon sources is analysed. Above all, if the three metabolic properties chosen by Alippi & Aguilar (1998) are variable features within the genotypes it will be impossible to find any obvious linkage between both features.

Based on the API 50CHB system, Carpana *et al.* (1995) determined the ability of *P. l. larvae* to metabolize 49 carbohydrates and their derivatives and presented a detailed comparison of the results with those reported in the literature. In particular for galactose, fructose and mannitol the results were contradictory. We found that among the isolates investigated in our study only genotype *AB* was able to metabolize D-fructose and mannose, for example. Therefore, we propose that the discrepancies between different studies are in part due to different genotypes analysed in these studies that in turn might be due to the differential geographic origin of the isolates.

Our data show that genotype *AB* is outstanding in respect to colony morphology and metabolic fingerprint. When screening all isolates used in this study for extrachromosomal DNA, only representatives of genotype *AB* were found to harbour plasmids. So far, no plasmid DNA has been detected in isolates from genotypes *Ab*, *ab* or *aβ*. Therefore, another characteristic feature of genotype *AB* is the presence of plasmids. The occurrence of plasmid DNA in *P. l. larvae* has been reported (Benada *et al.*, 1988; Bodorova-Urgosikova *et al.*, 1992; Drobnikova *et al.*, 1994). The plasmid denoted pBL423/728 is about 9·4 kb in size and this seems to be in agreement with our data at first. But whereas plasmid pBL423/728 does not contain an *Xba*I restriction site and digestion with *Eco*RI results in two fragments of 3·6 and 5·8 kb (Bodorova-Urgosikova *et al.*, 1992), plasmid pPll9.4 characterized in our study is linearized by *Xba*I and gives rise to two *Eco*RI fragments of 850 and 8550 bp. Hence, the two plasmids, although similar in size, are not identical. Since pPll11.0 differs from pPll9.4 only by a 1600 bp insertion, this plasmid also is not related to plasmid pBL423/728.

It has long been recognized that the proteins comprising the parasporal Cry toxins of *Bacillus thuringiensis*, an insecticidal, Gram-positive, spore-forming bacterium, are generally encoded by large plasmids (Gonzalez *et al.*, 1981; for review see Schnepf *et al.*, 1998). Nothing is known so far about toxins expressed by *P. l. larvae*. It will be interesting to screen the newly found plasmids of *P. l. larvae* for genes possibly involved in pathogenicity.

In some countries, *P. l. larvae* has been treated in bee colonies by the antibiotic oxytetracyclin for several decades. Recently, widespread resistance to oxytetracyclin has been reported (e.g. Miyagi *et al.*, 2000). A recent study analysing the origin of oxytetracyclin resistance in *P. l. larvae* did not include any search for plasmids, but rather focused on the correlation between 16S rDNA haplotypes and resistance (Evans, 2003). No convincing correlation was found, thus leading to the speculation that resistance might be epigenetic in nature, specifically through the presence of plasmids and mobile genetics entities that produce proteins involved in resistance (Adams *et al.*, 1998). Although no specific resistance to sulphonamides, antibiotics, mercury chloride or cadmium nitrate connected with the presence of plasmid pBL423/728 in *P. l. larvae* was found (Benada *et al.*, 1988), this is still an open question for pPll9.4 and pPll11.0. Since both these plasmids are not related to pBL423/728 it will be interesting to further characterize the newly identified plasmids and look for any resistance-connected genes carried by those plasmids.

Overall, our study has identified and characterized the exceptional *P. l. larvae* genotype *AB* for the first time. Considering what is known so far about this genotype, it may be the first choice for more detailed analyses with respect to virulence, pathogenicity and antibiotic resistance.

ACKNOWLEDGEMENTS

This work was supported by grants from the ministries for agriculture from Brandenburg and Sachsen-Anhalt, Germany.

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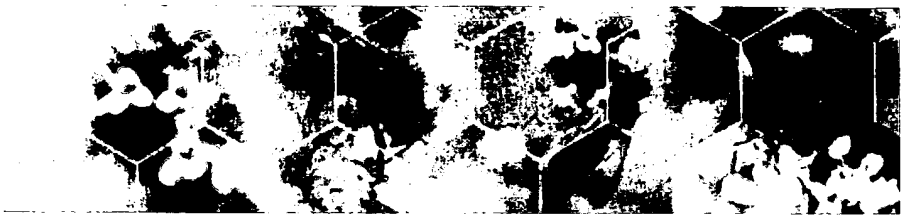
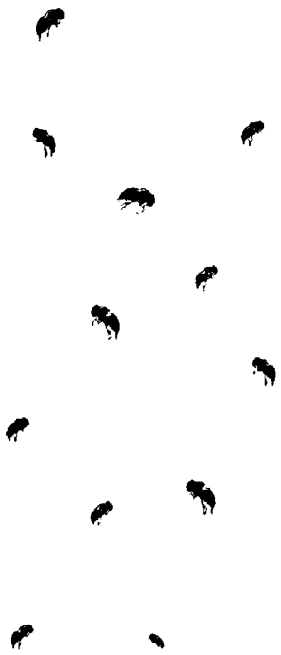




American Foulbrood

Introduction

American foulbrood (AFB) is the most serious, contagious notifiable bacterial disease of honeybee brood of international proportion. The causative organism is a virulent spore-forming bacterium, Paenibacillus larvae var. larvae. AFB can appear and spread quickly through a colony and if left untreated may result in the death of the hive in a short space of time.

Infection

As with many disorders, apparition of disease is exacerbated with supplementary stress conditions such as lack of food, water, space or additional disease or pest attack. P.larvae var larvae forms spores which are resistant to desiccation and to antibiotic treatment. Spores can remain dormant for many years on hive and beekeeping equipment and in honey or wax. AFB spores can readily be transported and transferred by bees or through the unwitting manipulations of the beekeeper to new colonies.

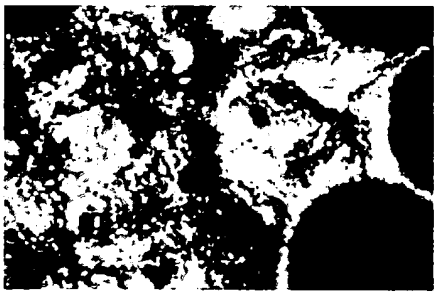
Young bee larvae become infected by Paenibacillus larvae var. larvae spores which may be already present in the cell, from housecleaning bees or through contaminated brood food. Once inside the larval gut the spores germinate and the bacteria multiply rapidly, moving from the gut into the surrounding tissues of the bee.

Bacterial proliferation is so great and so fast that infected larvae die within a few days, usually after the cells have been capped. The cadaver dries to form an infective "scale" in the bottom or on the side of the cell.

Untreated, an American foulbrood infection will spread rapidly through a colony killing much of the bee brood. At the end of a season this could result in a small, weak population to constitute the wintering colony.

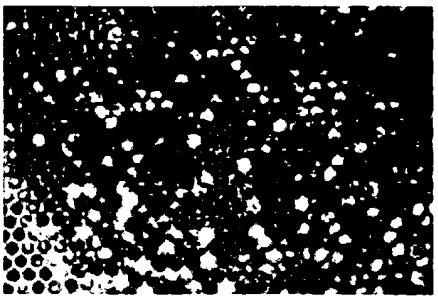
It is also at this time that AFB can be spread by robbing of the weak, infested colonies by bees from stronger, more healthy hives. Swarms from infected colonies may also transport the disease to new locations. Each scale resulting from American foulbrood infection will yield millions of infective spores.

Diagnosis

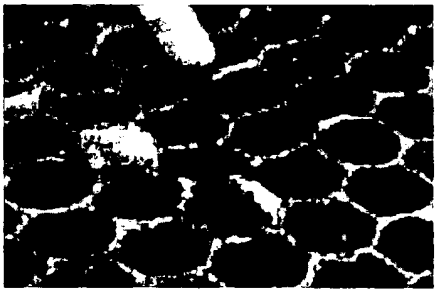


Signs of American foulbrood infection can appear at any time of the year. It is vitally important to detect AFB as early as possible. Vita has developed with Pocket Diagnostics a quick and effective Vita Diagnostic Test Kit. This enables every beekeeper to test their hives at the first suspicion of the presence of AFB. In severe infections the colony exudes a foul, rotten odour (hence foulbrood). Cell cappings may be dark and greasy-looking, sometimes sunken and perforated where the housecleaning bees have attempted to open them.

As in European foulbrood (EFB) infections,



the brood pattern in AFB-affected colonies can appear very patchy and irregular, sometimes termed "pepper-pot" in distribution. The queen will not lay eggs in cells previously contaminated with American foulbrood so the subsequent brood may be scattered sparsely over the combs in the remaining "clean" cells.



Bee larvae infected with American foulbrood die in the late larval stage with the body stretched out. As the cadaver decays (later drying to a dark sticky "scale" which is difficult for the housecleaning bees to remove) it becomes gelatinous.

If probed with a matchstick or similar implement the body can be "roped-out" which is a key diagnostic symptom of AFB disease.

Treatment

Good husbandry in beekeeping practice will help reduce other stresses on the honeybee colony and possibly limit the extent of any American foulbrood infection. In some countries, such as UK and New Zealand for example, the policy is to destroy colonies infected by American foulbrood by burning. This absolute measure in the UK has reduced the incidence of AFB dramatically.

In many other countries, administration of the antibiotic oxytetracycline (also known as terramycin) is permitted as a preventative as well as a curative treatment.

However, P. larvae spores can survive antibiotic treatment so application of antibiotic can only serve to suppress the vegetative stage of the bacterium. Spores can survive on combs, on bees, in honey and on any associated beekeeping equipment to reinfect the following or subsequent season. In North and in South America, bee colonies are often treated routinely with oxytetracycline, whether the colonies are overtly infected or not as American foulbrood is endemic practically world-wide.

Difficulties associated with this type of regime include cost to the beekeeper, residues of antibiotics in hive products, effect on the bees and more recently the emergence of oxytetracycline-resistant bacteria.

Ongoing Research

Vita (Europe) Limited in conjunction with the National Bee Unit and Cardiff University have won UK government funding for a research project evaluating a new biological control agent for foulbrood. A harmless bacterium found as a commensal in beehives has been shown to control Paenibacillus larvae var. larvae (as well as Melissococcus plutonius) infections under laboratory conditions. Studies on the toxicity and palatability of the bacterium show no effect on the bees.

Field trials against both European foulbrood and American foulbrood are underway and current progress is positive.

A new, natural product may be available from Vita (Europe) Limited for the treatment of foulbrood within

the next few years.

Research

Research Project: Sequencing of the Honey Bee Bacterial Pathogen, Paenibacillus Larvae and Fungal (Ascosphaera Apis) Genomes

Location: Honey Bee Research

Project Number: 6204-21000-009-03
Project Type: Specific C/A

Start Date: Jun 01, 2003
End Date: May 31, 2007

Objective:
The objective of this cooperative research project is to generate sequence coverage to assemble the genome of the important honey bee pathogen Paenibacillus larvae subsp. larvae. The gram-positive bacterium, Paenibacillus larvae subsp. larvae, causes the most devastating larval honey bee disease, American Foulbrood (AFB). The second objective of this research project is to generate sequence coverage to assemble the genome of the important honey bee fungal pathogen Ascosphaera apis. A. apis causes another devastating honey bee disease, Chalkbrood. Genetic sequences for P. l. larve and A. apis would provide the first opportunity for interactive whole genome analysis of the insect host and its natural bacterial and fungal pathogens. This amendment involves the sequencing of three plasmids from P. larvae subsp. larvae, which are estimated collectively to have a length of roughly 200 kb.

Approach:
The strategy developed at BCM-HGSC will aim for 10X coverage of the ~5 MB P.l.larvae genome. A short-insert plasmid library (1-3 Kb, randomly sheared) will be constructed from a virulent isolate of the bacterial pathogen collected from a single honey bee colony with severe AFB symptoms (isolate #230010, Berkeley, CA). Other libraries will be made to more precise specifications for the P. larvae genome project. A total of 120,000 sequence reads are predicted. In addition to the construction of the draft DNA sequence, initial genome annotation and publication of the P.l.larvae genome are

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expected. The same strategy will be implemented to generate 5.5X coverage of the ~(22-24) MB A. apis genome. Virulent isolates of the fungal pathogen were collected from a single honey bee larvae infected with the Chalkbrood. A short-insert plasmid library (1-3 kb, randomly sheared) will be constructed from a single mating type of the fungus, isolated from the original culture. The second plasmid library will be constructed to generate 2X coverage of the genome isolated from the second fungal mating type. An additional plasmid library would be constructed to generate 1X coverage of the P. larvae native plasmid DNA.

Last Modified: 06/11/2006

ANNEX D

DSMZ - List of Microbial Species: *Paenibacillus larvae* subsp. *larvae* (Bacteria) **DSMZ**

Name	***Paenibacillus larvae*** subsp. ***larvae*** (White 1906) Ash et al. 1994 emend. Heyndrickx et al. 1996 *VP* - see also Bacterial Nomenclature Up-to-Date
Synonym	***Bacillus larvae***
Restrictions	**Animal pathogen, restricted distribution (F)**
Strains	7030